2021 Animal Influenza Research Review

Compiled and written by

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Contents

Commissioning Body

This report was commissioned by the STAR-IDAZ International Research Consortium in collaboration with the Agricultural Research Service, United States Department of Agriculture.

The STAR-IDAZ International Research Consortium

The STAR-IDAZ International Research Consortium (IRC) is a global initiative aiming to coordinate research programmes at that international level and to contribute to the development of new and improved animal health strategies for priority diseases/infections/issues. The partners, research funders and programme owners together form the Executive Committee which is supported by a Scientific Committee of 16 experts and an EU-funded Secretariat (SIRCAH – Horizon Europe Grant Agreement Number 727494).

The target deliverables of the STAR-IDAZ IRC include candidate vaccines, diagnostics, therapeutics, other animal health products and procedures, and key scientific information/tools to support risk analysis and disease control. To achieve these goals the IRC partners agree to coordinate/align their research programmes to address identified research needs relating to the priority topics and to share results. Research gaps identified by expert Working Groups are organised into research roadmaps for the development of (i) candidate vaccines, (ii) diagnostics, (iii) therapeutics and (iv) disease control strategies, providing a structure to plot the identified research gaps and focus future investment (Entrican et al. 2021).

Agricultural Research Service, United States Department of Agriculture

The Agricultural Research Service (ARS) is the principal in-house research agency of the United States Department of Agriculture (USDA). ARS is one of four agencies in the Research, Education, and Economics (REE) mission and is charged with extending the nation's scientific knowledge with research projects in agriculture, human nutrition, food safety, natural resources, and the environment. ARS supports more than 2,000 scientists and post-docs organized into approximately 660 permanent research projects at over 90 locations across the country and five overseas laboratories.

ARS conducts innovative research to find solutions to problems of high national priority that impact the American people daily. ARS often undertakes high-risk research endeavors to make significant breakthroughs in important problem areas, including biodefense initiatives to detect, prevent, and mitigate the impact of especially dangerous infectious diseases that pose a threat to animals and

public health. As such, ARS organized the Animal Influenza Viruses Gap Analysis Workshop in Athens, Georgia, March 25-27, 2013, to assess the scientific information and countermeasures available to effectively control and mitigate the global impact of emerging animal influenza viruses.

Purpose of the Report

Effective control of animal influenza virus is a global goal that will require co-ordinated research efforts from scientists around the world. The success of these efforts relies upon funding of the right research at the right time, and this in turn requires regular analyses of what is known and unknown in the field.

The workshop reports conducted by the United States Department of Agriculture (USDA) and OFFLU (the OIE/FAO network of expertise on animal influenza) in 2014, and by the European Food Safety Authority in 2015 (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015), identified a panel of priority knowledge gaps across animal influenza research. The purpose of this report is to revisit that panel, report on progress in these areas, and to provide a general overview of the research that has been conducted across the major fields of animal influenza in the past six years. Further, by incorporating research updates and input from leading scientists in the field, this report represents a current picture of animal influenza research around the world, enriched by the first-hand knowledge of those working at its cutting edge.

The findings of the report will be used to support future detailed gap analyses that will also incorporate expert opinion and review of current research and control measures, alongside knowledge of on-the-ground countermeasures (both in use and under development) and their efficacy. Importantly, this literature review does not attempt to rank the knowledge gaps identified, and this will therefore form a key part of future analyses.

About the Authors

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Dr. Lucy Robinson gained her D.Phil. in veterinary immunology from the University of Oxford, UK, and continued her post-doctoral research into exotic infections at the Singapore Immunology Network. Following her editorial training and freelance experience, Lucy founded Insight in 2009 and has since successfully assisted with the publication of hundreds of articles and the construction of successful grant applications across diverse scientific fields. She has spoken at international conferences and delivered effective writing training to scientists from around the globe.

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Executive Summary

In this report we combine comprehensive literature review with input from leading researchers in the field (for details of contributors see here) to assess progress in animal influenza research globally since 2014/15. By reference to previously identified knowledge gaps and expert consultation, we provide a literature-based update that identifies some of the areas in which future research and research funding should be targeted for maximum impact. The gap analysis presented here is intended to be used as a tool to supplement future in-depth gap analyses that include additional factors.

This report comes at a timely juncture for zoonotic virus research, as the 2019/20 Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) pandemic continues its deadly course around the globe. The incredible progress made in our understanding of this novel virus and the disease caused by it in the past eighteen months is testament to the advances that can be made when governments and the scientific community pull together. As well as the specific points detailed below, the broader question now is what animal influenza research can learn from the successes of SARS-CoV-2 research, most notably the development of highly-effective outbreak monitoring and control procedures alongside the design, testing, licensing and large-scale production of several potent vaccines that are proving instrumental in protecting from clinical disease and reducing viral spread.

Research priorities by area:

Understanding Animal Influenza

Epidemiology

Increasing capacities for active surveillance, coupled with new sequencing technologies that allow rapid and complete whole-genome analysis, may facilitate a significant expansion of our ability to track and control novel influenza A virus (IAV) subtypes. In-depth studies continue to uncover the genetic and molecular determinants of pathogenicity, virulence, airborne transmission, and host tropism.

Some of the major difficulties facing the field of animal influenza epidemiology lie in the effective deployment of novel technologies, the integration of epidemiological data with new and existing national surveillance and control systems, and the ever-increasing global complexity of the influenza landscape. Our growing surveillance capabilities and understanding of biosecurity measures continue to come up against the limitations of knowledge and compliance levels among local poultry farmers,

who are often economically disincentivized to participate in biosecurity programs at a national level. From an economic standpoint, more research is needed to analyse biosecurity measures at the local-regional level and determine incentives that may raise compliance with surveillance/reporting programs and outbreak control measures.

To be ready for the next pandemic, ongoing surveillance is required to keep tabs on new patterns of viral evolution and epidemiology. Going forward, combinations of large- and small-scale studies will allow us to track the dynamics of major avian influenza virus (AIV) threats while keeping tabs on less well-understood viruses with pandemic potential.

Avian

The past five years have seen several significant avian influenza outbreaks and the continuing evolution and diversification of the viral landscape. Highly Pathogenic Avian Influenza (HPAI) H5Nx clade 2.3.4.4 has spread to countries across the world and caused major outbreaks in Asia and the United States. H7N9 has been the cause of outbreaks in China, with the last wave including a marked uptick in human infections and the evolution of a high pathogenicity phenotype. Research and surveillance are constantly required to track new and developing influenza strains in wild and domestic bird populations. In combination with molecular and virological studies, such research may allow the identification of viral subtypes of particular concern (i.e. expressing molecular patterns associated with increased virulence, viral replication, and/or cross-species transmission) and help to focus limited resources where they are likely to be of greatest benefit.

An emerging field of research covers the impact of anthropogenic and climatic factors on avian influenza evolution and spread. Meat production levels continue to increase worldwide with a commensurate increase in domestic poultry density and number of densely-populated sites (e.g. live bird markets, commercial poultry farms, etc.). Meanwhile, the warming climate introduces new variables into the transmission patterns of avian influenza and the interactions of domestic birds with their wild migratory counterparts. Knowledge remains limited on the impact of anthropogenic factors (e.g. live poultry market development, inter-farm movement, and global trade) on avian influenza transmission patterns and the ways in which a changing climate might alter viral behaviour and epidemiology. More research is also needed on wild reservoirs of avian influenza and the pathways by which they may interact with domestic birds.

Cross-species transmission (particularly zoonotic transmission) is an area of great concern, and predicting the zoonotic risk of avian influenza strains remains difficult. New developments – e.g. the recently-reported human cases of H10N3 in China and HPAI H5N8 in Russia (discussed in more detail below) – illustrate the importance of linking epidemiological studies with molecular virology databases to increase our ability to predict and control potentially zoonotic influenza subtypes.

Swine

Similar research concerns (e.g. increasing farm density and uncertain anthropogenic and climatic impact) are of paramount importance for swine influenza. Unique considerations here include increased risk of mammalian-adaptive mutations and the potential of pigs to act as mixing vessels amenable to infection by swine, human, and avian influenza strains.

Reverse zoonosis (transfer of influenza viruses from humans to swine) is a significant source of swine influenza genetic diversity, and our knowledge here remains limited. More research is needed on the rate of zoonosis/reverse zoonosis events and the pathways by which such events may generate variants with increased mammalian adaptation.

The 2009 H1N1 pandemic swine influenza strain remains endemic in many swine populations, and reassortment events are known to occur between these viruses and other subtypes of concern. Greater integration is needed between research and the meat production industry along with increased coordination between epidemiological, surveillance, and molecular biology databases to ensure our ability to keep up with particularly dangerous swine influenza strains and their spread/transmission characteristics.

Other species

Equine influenza is often overlooked but may present an understudied epidemic threat. Markers of reassortment between avian and equine influenza viruses have been observed and the close contact between humans and horses raises the risk of potential zoonotic transmission events. A similar situation exists for influenza virus in companion animals, predominantly dogs and cats. As international movement of companion animals is relatively limited and no major zoonotic outbreaks have originated from canine- or feline- transmitted influenza viruses, their epidemiology remains an understudied field. As advances in virology expand our understanding of the molecular determinants of human adaptation, it will be necessary to continually monitor influenza viruses in companion animals for markers of zoonotic potential or mammalian airborne transmissibility.

More research is also needed into influenza D virus (IDV), often associated with the bovine respiratory disease complex. IDV has not yet exhibited pathogenicity in humans, but experimental airborne transmission has been observed, and the basic properties of the virus remain understudied. Our IDV knowledgebase must be enhanced to ensure readiness for potential gain-of-function mutations within permissive farm animal populations.

Virology

As novel IAV strains emerge, molecular studies will be required to uncover the genetic determinants of virulence, host adaptation, environmental survival, etc. Sequencing technologies are the foundation of these efforts and continue to develop. Technologies (e.g. "third-generation" sequencing) that allow rapid, accurate sequencing in the field, preferably without the need for extensive training or expert assistance, could dramatically expand our ability to characterise circulating influenza viruses and monitor their evolution.

Computational resources also continue to grow in scale and accessibility, with consequences for genetic modelling, protein interaction studies, and other virological research. The effects of individual amino acid substitutions on viral protein function and stability, for instance, are being studied *in silico* – it is critical that such computational studies be integrated with ongoing surveillance programs and *in vitro*/*in vivo* research to ensure real-world applicability and robustness.

Avian

Many recent studies have focused on HPAI H5Nx clade 2.3.4.4 viruses and the molecular characteristics underlying their virulence and transmission patterns. These include mutations to internal genes that alter shedding/replication patterns and in the haemagglutinin (HA) / neuraminidase (NA) proteins that alter their functional balance or change receptor binding preferences. H7N9 has also come under intense study, as various strains of this subtype have evolved high pathogenicity and human infectivity. Molecular research has uncovered candidate mutations potentially responsible for these changes, including enhanced replication and binding to mammalian cell surface proteins.

In vitro and *in vivo* studies have analysed mammalian adaptation and the mutations responsible for enhancing human-type receptor binding, airborne transmissibility, and other hallmarks of zoonotic strains. Other genetic studies have focused on migratory bird populations and the mutations

necessary for efficient transmission of novel IAV strains from wild birds to domestic poultry. As sequencing and molecular analysis technologies continue to develop, they will need to be integrated with expanding surveillance programs to ensure that our understanding of avian influenza evolution is as complete as possible.

Swine

Reassortment events in swine are a concerning source of genetic diversity that can lead to enhanced virulence and transmission in a permissive mammalian host. Recent studies have also focused on antigenic drift and the evolution of novel subtypes in pigs. Cross-species infectivity remains an active area of research – molecular and sequencing studies have uncovered mutations that increase the ability of avian viruses to adapt to swine hosts, and of swine viruses to adapt to humans/human model hosts (e.g. ferrets).

Other species

The virology of equine and companion animal influenza remains an understudied field. Recent research has found a set of mutations potentially contributing to the decline of H3N8 and replacement with H7N7 as the primary equine strain, while in companion animals, mutations responsible for alterations of receptor binding preference have been described.

Pathogenesis

Sequencing and mutational analysis studies have begun to uncover the molecular determinants of influenza virulence and immune evasion, transmission and shedding, host/tissue tropism, and other factors relevant to viral infection, but further research is needed. As molecular biology and computational technology continue to grow, we can begin to systematically address the complexity of influenza genetics and the combinatorial effects that mutations can have on pathogenic factors.

Avian

Identifying factors beyond the multi-basic cleavage site that differentiate low- from high-pathogenicity avian influenza strains is an important priority. A related avian-specific aspect of pathogenicity is the often-observed clinical resistance of wild ducks to HPAI strains that cause significant mortality in infected chickens. Certain poultry species such as turkeys, meanwhile, exhibit enhanced susceptibility, potentially contributing to the circulation of new poultry-adapted IAV

variants. Here, research extends beyond mutational analysis and into *in vitro* and *in vivo* studies of the interaction of influenza virus with host immunity and cell biology, and immunological studies have identified host factors that impact the progression of Low Pathogenicity Avian Influenza (LPAI) vs. HPAI infections in poultry. It is important to integrate virological research with other fields of study (e.g. immunology) to get a more complete understanding of the host and viral factors affecting pathogenesis.

Many recent studies have focused on the pathogenesis of transmission – mutations that enhance transmissibility between domestic poultry, for instance, or that increase respiratory/cloacal shedding levels. Such mutations can also behave in a combinatorial fashion, making large-scale datasets and computational analyses increasingly important.

Co-infection is another critical aspect of pathogenesis research, as influenza is regularly accompanied by other viral or bacterial infections that may lead to increased clinical burden and mortality. Recent studies have highlighted the degree to which co-infections are not merely additive – proteins encoded by one pathogen can increase the pathogenicity of the other, for instance.

Swine

In swine, our understanding of the mechanics behind reassortment and gain-of-function mutations has increased considerably, but much more remains to be learned. Particularly dangerous swine influenza strains like H1N1pdm09 continue to circulate widely, with reassortments capable of producing strains with cross-species or zoonotic potential. Mammalian adaptation is a major field of swine influenza pathogenesis research, with genetic and *in silico* studies finding new mutations that may contribute to enhanced replication or airborne transmissibility. Co-infection is widely studied in swine as well, and new *in vitro* systems have recently been developed that may aid in the study of swine influenza pathogenesis and mutational analysis.

Immunology

Interactions between influenza virus and the immune system of its host affect pathogenesis, viral evolution, transmission (and therefore epidemiology), and also define the parameters for effective vaccination against influenza. This interaction is complicated and multifaceted, both at the individual animal and the population level, but is absolutely critical for us to understand. Significant advances have been seen in recent years, notably in avian immunogenetics underpinning differential resistance, as well as in our understanding of important factors involved in innate immunity to AIV: in swine,

knowledge of the effects of maternally-derived antibodies (MDA) has increased, with implications for the use of existing vaccines and the development of novel ones. However, attempts to link immunological findings with the bigger picture of pathogenesis, transmission and the development of broad long-lasting immune memory are scarce, and the complexity involved invites the use of computer modelling approaches. Such tools are being applied successfully in epitope design within animal influenza vaccinology, and in other areas of influenza virus research, such as epidemiology and outbreak control, but to our knowledge have yet to be used to comprehensively represent the innate and adaptive immune response to the virus and how this relates to aspects of protection or transmission. The application of computational immunology to the animal influenza field should be considered a priority area.

Avian

Knowledge of host immunogenomics has increased substantially, but studies in inbred poultry lines may over-simplify to the extent that they poorly-resemble real-world conditions. Host genetics appears to play an important role in both innate and adaptive immune responses to influenza virus infection. Next steps will include translation of this basic research into strategies that might be applied into the commercial field setting by rationally “breeding-in” increased resistance to avian influenza, or -where acceptable – the use of genetic modification to generate more resistant breeds. Studies assessing the possible impact of host immunogenetics on vaccine responses are so far lacking.

Several studies have expanded the discussion around the differential susceptibility of ducks and chickens to HPAI, which was previously attributed almost entirely to RIG-I expression by ducks. Innate immune molecules most notably including members of the TRIM and IFITM families are pointing to additional pathways that contribute to determining susceptibility.

An emerging field of research concerns the effects of the respiratory and intestinal microbiota on host immunity in general, and on resistance/response to AI infection in particular. Alongside, numerous studies report immune-modulating effects of various dietary supplements: to our knowledge, there are not any studies linking these observations or dissecting the effects of immune-modifying diets on the microbiota. Given the potential for significant improvements to immunity rendered by modifications to the diet/microbiota of commercial poultry, greater focus should be placed on this area in the future.

Knowledge is still lacking on the chicken cellular immune response to HPAI and its significance during infection. This is in part due to a relative scarcity of high-resolution tools for analysis, but this limitation is beginning to be lifted, for example by the development of multi-parameter flow cytometry protocols and *ex vivo* organ culture systems. Exploring this aspect of the response will be a required foundation for future studies which must aim to link findings on innate, antibody and cellular immunity to AI in order to generate a comprehensive picture of the global response patterns that are linked with protection versus susceptibility.

Swine

Our depth of knowledge of the swine interferon response to IAV infection *in vivo* and *in vitro* at the molecular level has considerably deepened in recent years. However, studies looking to translate this knowledge into strategies to increase vaccine-induced protection are scarce.

Epitope prediction and identification approaches are supporting the findings of immunological observation studies that together indicate the presence of potentially cross-reactive T cells in pigs experimentally infected with SIV. Alongside, development of the inbred Babraham pig has increased the toolkit for immunological studies of cellular responses to infection. The translation of these findings into rational vaccine designs for testing in the field will be of great interest.

The effects of MDA are becoming clearer and it now seems that, while MDA reduce the transmissibility of SIV, the remaining level of transmission may remain sufficiently high to propagate the infection within the herd. However, as MDA have the potential to protect piglets and reduce transmission, it will be important to understand the upstream factors accounting for the differences in protection elicited in piglets from sows with comparable antibody levels. Strategies aiming to maximise and standardize protection by MDAs should be assessed as part of defining an effective vaccination program for both breeding sows and piglets that avoids the risk of maternal antibody interference with vaccine immunity.

Other species

The study of equine and companion animal immunity to influenza virus infection is in its infancy.

Immune correlates of protection remain elusive across all species: although it is clear that antibody/HI titre paint an incomplete picture, the other components of the protective response remain to be fully defined. The results of immunogenetic studies indicate that resistance to infection is a complex trait

that varies between breeds/species, and therefore a simple set of immune correlates may not be possible to define given the many variables surrounding natural infection and the individual response to it.

Controlling the disease

Surveillance

Surveillance programs monitor circulating animal influenza strains to allow rapid outbreak response, targeted vaccine development, and more effective deployment of limited diagnostic and disease control resources. Recent studies have focused on the development of data collection technologies, new computational/modelling-based approaches, and strategies for integration between surveillance programs worldwide. Advances have been reported in bioaerosol testing and environmental sampling, allowing easier and less invasive monitoring of circulating influenza strains at domestic farming facilities. Modelling strategies also continue to develop, with researchers applying computational power to transmission network models, outbreak risk predictive maps and other means of targeting limited resources to areas of highest risk.

Computational resources have also been applied to enhancing integration between surveillance and other fields of research (e.g. virology and epidemiology) and across national borders. Online databases are an increasingly popular means of storing surveillance data, allowing input and analysis from scientists across the world. Combining such networks with relatively simple, non-invasive methods of surveillance may increase disease reporting compliance and allow surveillance programs to cover previously understudied regions and/or species.

Diagnostics

Early detection in livestock or in wildlife populations of IAV subtypes with zoonotic and/or pandemic potential is a clear priority. To achieve this aim, reliable diagnostic tools for virus and antibody subtyping should be developed as alternatives to haemagglutination inhibition (HI). Both lab-based, high-throughput tests and cost-effective point-of-use tests with minimal technological requirements are necessary. Similarly, although multiplexed methods that simultaneously test for many or all of the known (and even unknown) IAV subtypes can fulfil an important role in viral surveillance, there are

also situations that would benefit from subtype-specific tests. To enable comparison of diagnostic-test results between laboratories, appropriate internal controls should be developed.

Viral antigenic variation presents a challenge for the continued effectiveness of established diagnostic tests. Because host range, pathogenicity and zoonotic potential are dependent upon the subtypes of the viral HA and NA proteins, assays that can accurately determine the HA/NA status of identified IAVs are required. Once developed, such assays should be monitored to ensure their continued effectiveness in the context of IAV genetic mutation and antigenic drift. Viral genomic sequencing should be conducted to ensure that the available tests provide full coverage of the circulating viral pool, and it can be facilitated by improvement of methods for virus recovery and storage. In addition, tests that are not affected by mutations in the *NA* and *HA* genes, such as those that target conserved IAV genes and proteins, should be used where appropriate.

Ongoing and past IAV infections can be identified by appropriate profiling of serum antibodies, either by the standard method of HI or by alternative techniques such as ELISA. The available assays should be revised and updated to ensure that they are suitable for identification of infection with the currently circulating IAV strains (and that they can determine the subtype specificity of the serum antibodies), and novel approaches should be developed to improve assay precision, multiplexing and suitability for point-of-use testing. In addition to the direct identification and characterization of circulating IAVs, serological testing can characterise humoral responses to vaccination, and has the potential to differentiate between antibody profiles resulting from vaccination and from infection. Appropriate DIVA strategies should therefore be developed.

Vaccines

Successful control of animal influenzas globally will most likely require the use of vaccines. Current vaccines can be efficacious under optimal conditions of close antigenic match with the circulating strain, precise administration and adherence to the recommended vaccination schedule, lack of co-infection with other pathogens, absence of immunosuppression, favourable (as yet undefined) immunogenetics of the host, and no interference from maternally derived antibodies. In short, there are significant barriers to efficacy of many currently available vaccines.

Avian

A major stumbling block remains the widespread use of vaccine production methods that rely upon inoculation of viral strains into vast numbers of embryonated eggs from chickens. This is highly problematic on multiple levels: the time between new strain identification and vaccine availability is typically four to six months, partly due to the need to adapt strains for growth in eggs, which in itself can increase the antigenic distance between the field and vaccine strain and so reduce vaccine efficacy; moreover, the supply of eggs could clearly be adversely impacted in an AI epizootic and/or pandemic situation. At best, production of AI vaccines in eggs could be considered out-dated, while at worst it is a ticking timebomb leaving us vulnerable to a potentially catastrophic inability to manufacture AI vaccines in response to an emerging threat.

Moving away from egg-based methods of AI vaccine production is clearly desirable and probably essential, but there is currently little incentive for vaccine companies to go through costly and time-consuming regulatory processes and invest in new infrastructure for different methods. Similarly, many novel vaccines are being described in the literature that continue to require propagation in embryonated eggs. Other methods of vaccine production are widely described, and research should be encouraged that brings together the best in vaccine design with forward-thinking approaches to subsequent vaccine production such as the use of suspension cell cultures or plant-based methods.

Also urgently needed are new technologies for mass vaccination, particularly critical for the broiler industry, to facilitate influenza control in densely-populated poultry environments where avian viruses can spread rapidly if left unchecked.

Swine

There are promising novel vaccines emerging from SIV researchers globally, including those based on DNA and live-attenuated viruses. Intriguing data are being reported on the potential for heterologous prime-boost immunization protocols to increase protection, also hinting at the presence of little-understood immune mechanisms underlying breadth and strength of protection that warrant further investigation. Computer modelling is being used to drive rational vaccine design, with encouraging results, calling for more widespread use of these approaches across animal influenza vaccine research.

Other species

Research into current and novel vaccines against equine influenza is ongoing, including important insights into the effect of maternally-derived antibodies.

A canine influenza vaccine for use in cats is warranted.

All species

Previous reports have called for the design of novel vaccines across all species and the research community has responded positively, with many candidate vaccines showing significant promise. However, previous reports also identified the lack of field-testing of vaccines as a significant problem, and this issue remains widespread. Similarly, studies variably report on innate immune responses, serum or mucosal antibody and/or cytokine levels, cellular immunity, duration of immunity, comparisons with inactivated vaccines, clinical protection from homologous/heterologous challenge and viral shedding at various time points, to name but a few parameters. This variability makes meaningful comparison between studies, and therefore between vaccines, a real challenge, and definition of an agreed-upon set of standard measurements for all AI vaccine studies (as seen in human vaccine trials) could be tremendously beneficial.

There is also a need for systematic testing of adjuvants across different species and, in particular, under field conditions. Adjuvants have the capacity to influence the antigenic dose necessary, time-to-protection, the titre and diversity of antibodies produced, the shape and magnitude of the T cell response, and the establishment of effective immunologic memory. However, they in turn are influenced by the amount used, the route of delivery and species-specific factors. Rarely are studies on adjuvants conducted in a systematic way that defines the optimal method/setting or their use, and the broad immune outcomes that result: rarer still are novel vaccines tested with a range of adjuvants or using different delivery routes.

Overall, across animal influenza vaccine research there is an urgent need for more “joining of the dots”, where novel vaccine candidates that show both practical and immunological promise are tested in a progressive manner and in depth, allowing for minor alterations to be made to increase efficacy, whilst moving towards the ultimate aim of demonstrating strong, durable, cross-protective immunity under field conditions. More support from funders and publishers for this type of approach will be

needed if the promising vaccine candidates of today are to be the answers the animal influenza outbreaks of tomorrow.

Drugs/Therapeutic approaches

Although neuraminidase inhibitors (NAIs) such as oseltamivir (Tamiflu) are available for prevention and treatment of viral influenza in humans, widespread use of these agents in animals is contraindicated on the basis of the cost of the drugs, the possibility of contamination of the environment and the food chain with the drugs and their metabolites, and, most importantly from the perspective of human health, the inevitability of the development and dissemination of viral resistance to therapy. Notably, however, such treatments have potential roles in specific aspects of animal health care, such as the protection of endangered species and valuable genetic stocks. Research is required to determine the appropriate use in these situations of existing anti-influenza drugs, whether approved or rejected for human therapy, and also to identify alternative treatments that are not subject to the limitations of known NAIs, and that are suitable for widespread use and not associated with adverse effects on human health.

Priorities for basic research in this field have also been identified as the improvement of resistance to IAV infection, either by selective breeding or by genetic modification, as well as the development of interventions to block viral replication. A promising direction for research in this field involves the development of agents that mimic host cell-surface receptors, which can potentially prevent the initiation of viral infections.

Disinfectants

The development of new disinfectants, particularly those with a minor environmental footprint, remains a priority. Equally important is the ability to apply such chemicals effectively under varying circumstances (farm layout, temperature, humidity, etc.) and to thoroughly clean them from the environment. Many recent studies have focused on disinfectants that are effective across a range of climatic conditions, while others have examined UV irradiation, air filtration/treated filters, and other broad-scale methods that may be relatively simple to deploy in diverse situations. Public outreach and education strategies should also be expanded to ensure understanding of and compliance with outbreak control strategies. Effective and efficient disinfection in the wake of influenza infections can

substantially reduce the time and cost required to recover from an outbreak, reducing economic burden in the long run.

Depopulation and Disposal

Culling and disposal of affected animals is the first step in influenza outbreak control. Many methods exist for this step; recent studies have primarily focused on making depopulation and disposal more effective and efficient, more humane, and less environmentally impactful. Euthanasia of layer/caged birds remains more challenging than for floor-reared birds due to the layout of such poultry farms, for instance, with methods such as fast-deploying foam currently under study. From an environmental perspective, composting is a disposal method with a lower footprint than burning or burying, but increased integration between farming and industrial sectors may be needed to make large-scale composting feasible in resource-limited regions.

Depopulation and disposal are expensive, and cost avoidance can reduce disease reporting/control program compliance in areas with little to no biosecurity. Economically-minded research has focused on reducing the necessary radii of depopulation and reducing the number of animals that must be culled to ensure effective outbreak control. Computational studies and *in silico* modelling may provide guidance for reducing the maximum necessary extent of depopulation during an outbreak, lowering the economic burden of culling and disposal and hastening post-outbreak recovery.

Personal Protective Equipment

Personal Protective Equipment (PPE) is a critical component of worker-protection in outbreak situations; however, risks of user-error, non-compliance with use and the effects of equipment wear and tear can limit efficacy. Recent studies provide encouraging data that show PPE can be effective, it is important to understand how PPE performs during longer durations of potential exposure and in viral strains with known high transmissibility to humans. Thus, future studies should report as much detail as possible on duration and intensity of transmission and include data on reported breaches of correct PPE use.

Literature Review and Research Updates by Subject Area

Report approach

The primary literature review was conducted using the applied life sciences CAB Abstracts database (www.cabdirect.org) and the search term "influenza*". When limited to studies published since the most recent previous gap analysis (July 2015), the database returned a list of 6818 papers. Papers were subsequently excluded from further consideration if they were not published in English or not relevant to animal influenza. The remaining papers were then manually screened for relevance to the scope of this report, limiting species of interest to all avian, swine, equine and companion animals, finally leaving 3562 papers for assessment for inclusion. These papers were allocated to the following topic areas as shown in

Table 1.

Research Category	**Papers (n)**
Epidemiology	1035
Virology	470
Pathogenesis	339
Immunology	233
Surveillance	175
Diagnostics	235
Vaccines	810
Drugs/therapeutic approaches	208
Disinfectants	34
Depopulation and disposal/Epidemic control	19
PPE	4
Total	3562

Table 1: Animal influenza peer-reviewed publications July 2015-March 2021, sub-divided by topic area.

These studies formed the main structure of the report and were supplemented by 70 recently published studies retrieved from the PubMed.gov database, which has a shorter delay between publication and upload of article information than CAB Abstracts does. Additional literature searches were performed during writing to provide appropriate citation for all material and, where needed,

useful background. Studies were selected for inclusion based on the authors' impressions of their potential impact within the field, their quality and novelty, and the relevance of their findings with respect to the knowledge gaps identified previously in 2014/15. More recent studies were given priority within the report. In total 682 studies are referenced herein.

To generate an overview of ongoing research, 143 animal influenza researchers and experts were contacted by email and invited to complete a survey that asked for brief summaries of their current and future projects in animal influenza research. These individuals were selected from attendance lists for the previous gap analysis workshops, personal connection with the commissioning team at STAR-IDAZ IRC, or through being among the list of most prolific authors within the "influenza*" search of the CAB Abstracts database (defined as having contributed to >35 publications published between 2015 and 2021 inclusive). Information provided by these researchers and experts is included within the appropriate report section, but it should be noted that the representation of work is thus inherently biased towards those individuals and institutes that elected to respond to the request for information. Therefore, these sections should not be considered comprehensive in the same way as the rest of the report aims to be.

Two strategies were combined to identify current knowledge gaps: the knowledge gaps identified in 2014/15 were assessed to establish whether they have yet been filled or remain in need of further research; secondly, the animal influenza researchers contacted for ongoing research updates were also asked to submit their thoughts on current research knowledge gaps. Current knowledge gaps based on the literature review and expert opinion are summarised at the end of each literature review section.

Introduction

Animal influenza viruses have been recognized as a threat to the health of wildlife and livestock since the 1800's. During the 1918 human flu pandemic, it was noted that swine also became ill and showed similar clinical signs, raising the possibility of transmission between species. Since this time, outbreaks of avian and swine influenza that have crossed into humans have claimed many lives, and predictions of the likely death toll of a pandemic in which an animal influenza virus gained the ability to transmit between humans vary from 5-150 million. The ongoing severe acute respiratory syndrome coronavirus 2 pandemic, which likely originated from a zoonotic infection in China, brings these figures into sharp focus and argues for strong and systematic research into viruses that pose a similar threat to human health.

Alongside their zoonotic risk, swine, avian, equine and companion animal influenza viruses have serious economic and social impacts as animal pathogens. For example, highly pathogenic avian influenza (HPAI) causes direct mortality in susceptible species, but outbreaks are often limited by culling of huge numbers of infected, suspected infected or uninfected at-risk birds in the area, which adversely affects commercial and backyard poultry farmers both financially and, in some cases, mentally, from the trauma of the culling itself (H. Park, Chun, and Joo 2020). Moreover, HPAI outbreaks can drive down consumer demand for poultry products for prolonged periods (Wen et al. 2019; Belewu 2019) and areas that rely on tourism can also suffer long-lasting adverse effects from outbreaks (Govindaraj et al. 2018). Swine influenza is rarely fatal but causes morbidity within affected herds, especially when it occurs as part of the porcine respiratory disease complex, leading to fewer piglets and longer time to slaughter in endemic herds (Donovan 2005) while the recent equine influenza outbreak in the UK in 2019 was estimated by the media to have cost the horse racing industry alone over £200 million.

The combined risk to human and animal health from influenza virus infection calls for a research framework that strategically aims to answer key questions around the biology of the virus, its host species, their interactions, and how these factors combine in outbreak situations. Building on this knowledge we can then establish optimal tools for control and use retrospective analysis and outbreak modelling to understand how best to apply them. This report provides updates on these key aspects of animal influenza virus research and control and aims to establish those avenues of future investigation that will most effectively propel us towards improved prevention and management of influenza outbreaks in animal species, with the parallel aim of preventing crossover into the human population.

Understanding Animal Influenza

Epidemiology

The incidence and distribution of veterinary influenza has grown ever more diverse over the past 5 years. Previously recognized influenza viruses continue to disperse across the globe, increasing in complexity as they encounter new host species and reassort to form novel virus strains. At the animal-human interface, the rapidly increasing human population has necessitated an explosion in food animal production, with poultry meat production alone increasing by 21.3 million tonnes from 2010-2017 (Hautefeuille, Dauphin, and Peyre 2020). This has resulted in a higher density of farm animals and animal species, facilitating the transmission and reassortment of new influenza strains that regularly cause costly outbreaks in developed and developing nations (Ruiz-Fons 2015).

Over the past five years, the highly pathogenic influenza subtype H5Nx has remained a significant cause of outbreaks in domestic poultry and continues to spread across the globe via spillback from poultry to wild migratory birds (Verhagen, Fouchier, and Lewis 2021). H7N9, previously established as a significant threat in China, was observed in Guangdong during 2016-2017 to evolve from a low pathogenic to a highly pathogenic phenotype, with a coincident increase in cases of zoonotic infection (Shuo Liu et al. 2020). Meanwhile, other potentially zoonotic viral subtypes like H9N2 continue to circulate, particularly in Southeast Asia, a long-recognized hotspot for poultry influenza reassortment within its loosely regulated live bird markets (Peacock et al. 2019).

In swine, a "mixing vessel" species susceptible to infection by both avian and human influenza viruses, new patterns of viral reassortment and epidemiology have been observed worldwide. In Europe, swine populations have been found to harbour multiple major influenza lineages of the H1 and H3 subtypes; intensive reassortment continues to generate distinct genotypes, some with pre-pandemic properties (Henritzi et al. 2020). In developing countries, meanwhile, concerning subtypes including avian H9N2 and H5N1 and the human-origin H1N1 2009 pandemic strain have been found co-circulating in domestic pigs in developing countries (Gomaa et al. 2018; Meseko et al. 2018). Zoonoses and reverse zoonoses also increase the probability of highly transmissible reassortants in developed nations (Nelson et al. 2020; Henritzi et al. 2020; H. Sun et al. 2020). A plethora of other species serve as reservoirs for the circulation and evolution of influenza viruses, with horses (Diallo et al. 2021) and companion animals (Kalhoro et al. 2019) being of particular interest due to their close proximity to humans.

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps in animal influenza epidemiology in 2014/15:

- *understanding patterns of host susceptibility based on molecular markers*
- *identification of critical reservoir species, particularly within wild bird populations*
- *genetic determinants of resistance to HPAI strains in known reservoir species (e.g. ducks)*
- *factors impacting speed of antigenic drift and shift, especially in swine*
- *interaction pathways between wild birds and poultry, and computational models of such pathways*
- *modes of environmental transmission*
- *increasing understanding of live poultry markets and related viral transmission dynamics*
- *determinants of pathogenicity including and beyond the multibasic cleavage site*
- *determinants of host and tissue tropism*
- *mechanisms of mammalian adaptation in avian viruses*
- *role of environmental and ecological variables in virus transmission patterns*
- *increasing understanding of potential reservoir species at the human/animal interface*
- *understanding of frontline worker biosecurity measures and means for increasing compliance*
- *mathematical/computational modelling of outbreak dynamics to inform public policy*

Literature review

Epidemiology of Avian Influenza

After classification based on their hemagglutinin (HA) and neuraminidase (NA) surface proteins, avian influenza viruses (AIV) are divided into low pathogenicity AI (LPAI) or HPAI categories based on their phenotypic presentation in chickens. LPAI viruses may circulate with minimal or even no clinical signs, allowing long-term, unobserved transmission and maintenance in domestic flocks and wild bird populations. However, some LPAI viruses may switch categories by spontaneously acquiring the defining hallmark of an HPAI strain: a multibasic cleavage site (MBCS) within the HA protein that facilitates wider tissue tropism within the host (Beerens et al. 2020; Joseph et al. 2017; Stech et al. 2015). HPAI strains cause significant disease in domestic poultry and can cause up to 100% mortality

in infected flocks. Such outbreaks are therefore highly visible and usually quickly contained (W. S. Liang et al. 2020; S. H. Kim 2018). In regions where HPAI viruses are endemic and/or widespread, however, HPAI outbreaks can spread rapidly, usually from October through February in the northern hemisphere before tapering off as temperatures rise (Awada et al. 2018).

Both environmental and anthropogenic factors play significant roles in the spread of AIV. LPAI and HPAI viruses can spread between domestic poultry and wild migrating birds. These birds, particularly wild ducks, are often asymptomatic even during infection with HPAI and can maintain and transmit these viruses across their migratory routes. As discussed below, research continues to expand our understanding of avian influenza epidemiology within wildlife populations, revealing new host species and viral reservoirs and updating assumptions on the nature of AIV transmission within and between continents. Meanwhile, high-density domestic poultry sites (e.g. live bird markets and commercial poultry operations) and poorly regulated contacts between farms (e.g. movement of infected birds or contaminated equipment) also contribute to the transmission and inter-species reassortment of AIV.

Current Global Situation

Globally, the volume of reported avian influenza outbreaks in the 21st century has likely already exceeded the sum total from the 20th century (Canavan 2019). Recently, two particular subtypes of avian influenza have attracted significant attention due to their high pathogenicity, widespread distribution, zoonotic potential, or combination of the three. HPAI H5Nx viruses have long been recognized as a major source of poultry epidemics and occasional zoonoses in regions of Asia and Africa. In the latter, H5Nx have appeared to reach the continent via Europe via migratory waterfowl, and circulation/persistent infections in Africa may serve as a source of re-transmission to Europe, also via migratory birds (Fusaro et al. 2019). These H5 viruses have continued to spread rapidly over the past five years, with the particularly concerning clade 2.3.4.4 detected in poultry populations across the Middle East and Asia and in at least 19 European countries (Verhagen, Fouchier, and Lewis 2021). This clade has caused epidemics worldwide, with a 2014-2015 outbreak in the midwestern USA leading to the culling of >50 million poultry and a cost exceeding 3 billion USD – the largest outbreak of an HPAI in the continent's history. Meanwhile, H7N9 caused repeated seasonal epidemics of poultry influenza in China, with its fifth wave (2016-2017) including a 340% increase in human infections and an evolution to a highly pathogenic phenotype (Shuo Liu et al. 2020).

H5Nx Avian Influenza Viruses

The ancestor strain of the present HPAI H5N1 subtype emerged in Guangdong, China in 1996 and was initially restricted to Southern China (Dhingra et al. 2016). Within a decade, it had spread throughout the country and circulated widely in Chinese live poultry markets (LPMs) for at least 15 years. Prior to 2012, the most common subtype by far was H5N1. Thereafter, the H5N2, N3, N4, N5, N6, and N8 subtypes emerged and spread rapidly through the poultry population (Shuo Liu et al. 2020). The dominant subtypes have shifted rapidly over the past five years, and in the winter of 2020-2021, multiple outbreaks of H5N8 were reported across Europe and Asia (Jiahao Zhang et al. 2021).

Viral evolution in these regions led to the emergence of a diverse field of clades sharing the H5 gene (collectively terms "H5Nx" viruses). The dominant clade of the H5Nx viruses has changed over time, with clade 2.3.4.4 emerging in early 2014 and now accounting for 99.2% of Chinese isolates (Shuo Liu et al. 2020). HPAI H5Nx clade 2.3.4.4 has since spread worldwide at an unprecedented pace, reassorting with AIV in disparate regions to gain different NA while retaining the clade 2.3.4.4 HA (Dhingra et al. 2016). H5N8 spread from China to South Korea, Japan, Russia, and Europe within a year, with long-distance migratory birds playing a significant role in this spread (The Global Consortium for H5N8 and Related Influenza Viruses 2016). In South Korea, domestic ducks maintained the virus and passed it to poultry farms over a two-year period of continuous outbreaks (J. H. Kwon et al. 2020). Meanwhile, the novel reassortant H5N6 clade 2.3.4.4 circulated through Southeast Asia. H5 clade 2.3.4.4 reached North America by late 2014, most likely carried via migratory birds passing through the Beringian Crucible (D.-H. Lee et al. 2015). October 2016-August 2017 saw the largest recorded HPAI epidemic in Europe, including >1200 outbreaks in poultry holdings across 24 EU countries, with numerous reassortments occurring between HPAI H5 viruses and cocirculating LPAI viruses (Lycett et al. 2020).

An H5N2 outbreak subsequently developed in western Canada and the American Pacific Northwest, causing sporadic infections in domestic and wild birds until a sudden explosion in cases in the midwestern USA in March 2015. This stage of the outbreak lasted for three months and became the largest and most expensive HPAI outbreak in the USA to date, involving the culling of approximately 50.4 million poultry and an estimated loss of 3.3 billion USD across the economy at large (Hicks et al. 2020). H5Nx viruses expanded their spread across Europe, Asia, and Africa during 2016-2018 and now circulate worldwide (Salvador et al. 2020; Kouam, Tchouankui, and Ngapagna 2019; Adlhoch et al. 2020), threatening domestic poultry flocks and raising the likelihood of reassortment events between other endemic avian and swine influenza viruses (Jeong et al. 2020).

Although less hazardous to poultry than their HPAI counterparts, low pathogenicity H5 viruses continue to circulate and reassort as well. Li *et al.* examined the evolutionary dynamics of LPAI H5 viruses in North American domestic poultry and found evidence for 18 discrete introductions into various holdings from wild birds, facilitating cocirculation of multiple subtypes and raising the probability of reassortment (L. Li et al. 2018). In Mexico, vaccination programs and other control measures led to the extinction of HPAI H5N2 in the mid-1990s, but LPAI H5N2 has remained enzootic for decades despite significant containment efforts, with antigenic drift events facilitating vaccine escape (Bertran et al. 2020). Mexican-lineage LPAI H5N2 was introduced to Taiwan in 2003, where it reassorted with local H6N1 and mutated to form an HPAI variant responsible for poultry outbreaks in 2012 (Chung et al. 2020a). Infections and subsequent genetic divergence has also been detected in the Dominican Republic (Chung et al. 2020b). Reassortment between LPAI and HPAI H5 subtypes can be particularly dangerous, as low-severity disease can allow intercontinental transport of HPAI internal genes to go unnoticed (Lycett, Duchatel, and Digard 2019).

H7N9 Avian Influenza Virus

The H7N9 lineage that was widespread in China in recent years was first identified in March 2013, when human infections were recorded in the southeast of the country (Z. Zheng et al. 2019). This virus originated from AIV reassortment events between domestic poultry and wild birds (Artois et al. 2018), and unlike H5Nx, H7N9 emerged as an LPAI virus that caused minimal clinical signs in chickens; however, it seems to be a significantly higher zoonotic risk. Although the first wave of LPAI H7N9 was seemingly averted, a second wave began in late 2013 and led to 318 human cases and over 100 deaths (Lam et al. 2015). Once endemic in LPMs, the close proximity of multiple poultry species allowed rapid reassortment of H7N9 with spread throughout the country via the meat trade (D. Wang et al. 2016). As the virus spread, it diverged into numerous distinct clades, causing two subsequent epidemic waves with reduced zoonotic incidence. However, the fifth epidemic wave of LPAI H7N9 in 2016-2017 reached a record level of spill over into humans (Artois et al. 2018), combined with a westward spread that carried the virus out of its usual endemic zones in southeast China (Lau et al. 2019). Altogether, since 2013, H7N9 has caused more than 1500 human infections including 30-40% fatalities (Shuo Liu et al. 2020).

The virulence of the fifth H7N9 epidemic wave focused considerable international attention on this unpredictable threat. Genetic and virological studies revealed that H7N9 is better adapted to the

human nasal passages and respiratory system than any other known AIV subtype and is capable of limited airborne transmission between mammalian models (Peiris et al. 2016). Particularly unfortunate was the emergence of an HPAI H7N9 variant during the fifth wave, increasing the disease burden on the poultry industry until widespread vaccination assisted in halting the epidemic in April 2017 (Shuo Liu et al. 2020; S. Su et al. 2017).

No further substantial outbreaks of H7N9 have been reported since 2017, but the virus continues to circulate asymptomatically in poultry, with the ecologically rich Yangtze and Pearl River Deltas serving as H7N9 maintenance sites. It is highly seroprevalent in Chinese poultry workers, with an estimate of anywhere from 13 to 225 mild infections for each case requiring hospitalization (Peiris et al. 2016). Vaccination control programs are ongoing, but recent surveillance data suggests that vaccine pressure is accelerating the evolution of Chinese-lineage H7N9 – stricter control measures may therefore be necessary (Y. Wu et al. 2021a). Separate lineages of H7N9 have also caused outbreaks in the Netherlands and the United States (Sanhong Liu, Ruan, and Zhang 2017). In 2017, an HPAI variant of H7N9 was detected in two poultry farms in Tennessee (D.-H. Lee et al. 2017), most likely as an offshoot of undetected LPAI circulating within the population (Beerens et al. 2020).

Other Avian Influenza Viruses

H9N2 has circulated in the Chinese poultry industry for decades despite widespread vaccine use. It seems to be almost entirely sustained in domestic populations, with a high prevalence in chickens and pigeons and little in wild birds, and despite its limited transboundary potential, it remains substantially seroprevalent in poultry workers (Shuo Liu et al. 2020). H9N2 has spread across the globe in the years since its identification; different lineages of this virus are endemic in Iranian LPMs (Fallah Mehrabadi et al. 2019) and circulate in many regions including Bangladesh (Turner et al. 2017), Pakistan (Chaudhry et al. 2020), the United Kingdom (Reid et al. 2016), and many other areas of Europe, Asia, and the Middle East. H9N2 alone typically causes relatively mild respiratory disease in infected poultry, but co-infection with bacterial or viral pathogens can raise mortality rates to levels associated with HPAI viruses, leading to substantial economic burden in affected regions. Vaccination programs have been implemented across Asia, the Middle East, and Africa, but H9N2 continues to circulate and cause outbreaks in vaccinated populations (Peacock et al. 2019). Active surveillance/epidemiology studies, more regular vaccine strain assessment, and improved vaccination technologies are needed to reduce the risk of these viruses.

H6N1 and H10N8 AIV also circulate widely in poultry in China (S. Su et al. 2017). These strains emerged in 2015, most likely via reassortment of the dominant H5, H7, and H9 strains with viruses introduced by wild birds (Lam et al. 2015). Lloren *et al.* note that these and other H6 and H10 strains display phylogenetic attributes associated with mammalian adaptation, and both have been associated with limited zoonotic outbreaks in Southeast Asia over the past decade (Lloren et al. 2017). On June 1, 2021, China's National Health Commission reported the first known human case of H10N3 avian flu in the Jiangsu province, though there is no indication that this virus can spread easily between humans (H. Gu and Patton 2021).

In Mexico, LPAI H5N2 and HPAI H7N3 viruses are endemic in poultry. The latter has been responsible for recurrent outbreaks in Mexico since 2012 along with separate outbreaks in Chile and Canada. Vaccination control programs are ongoing, but a high rate of genetic divergence has allowed the virus to remain in circulation (Sungsu Youk et al. 2019). Adaptive mutations that have facilitated its pathogenicity in chickens, however, appear to have reduced its fitness in mallards, potentially limiting viral spread via wild waterfowl (S.-S. Youk et al. 2019).

Seroprevalence studies have highlighted the frequency with which multiple strains of AIV may co-circulate, increasing the risk of gain-of-function reassortment events as viruses are shared among species-diverse domestic and wild populations (discussed in more detail below). H1N2 and N3, H3N6, H4N2, H9N2, and H10N7, for instance, have all been detected within LPMs in Bangladesh (Biswas et al. 2017), and similar conditions exist in many regions throughout Southeast Asia and the Middle East where high-density live bird markets are common (Verhagen, Fouchier, and Lewis 2021; Khan et al. 2018). Meanwhile, small-scale and case studies allow monitoring of the spread of rarer virus subtypes not yet associated with significant outbreaks or zoonotic potential. In Colombia, H11N2 was observed circulating in an LPM in 2016 (Jiménez-Bluhm et al. 2016), and in 2020, Liu *et al.* reported the first isolation of H12N2 virus, originally detected in Canada, from wild birds in China (J. Liu et al. 2020).

Modes and Routes of Transmission

The primary modes of AIV transmission between infected birds are the oropharyngeal (respiratory) and cloacal (faecal/oral) routes, with the relative importance of these routes to transmission depending on the influenza strain and host bird species (Verhagen, Fouchier, and Lewis 2021; Pantin-Jackwood et al. 2017). In 2016, Ruiz-Hernandez *et al.* examined the importance of both modes using an H7N7 AIV to probe viral transmission characteristics in two chicken lines: one with an MHC/B

haplotype suggested to increase influenza resistance, and another with decreased resistance. They found that resistant chickens shed virus only via the oropharyngeal route unless specifically infected via the cloaca, suggesting an intrahost transmission barrier between the respiratory and gastrointestinal systems. Resistant chickens also caused significantly less viral transmission to naïve birds, while more vulnerable chickens spread the virus far more quickly via cloacal shedding (Ruiz-Hernandez et al. 2016).

Anthropogenic factors can multiply the risk of respiratory and cloacal transmission by providing opportunities for viral evolution and spread. LPMs are a particular mainstay of agricultural industries in China, Southeast Asia, and areas of Africa and the Middle East (Offeddu, Cowling, and Peiris 2016; Biswas et al. 2017; Chaudhry et al. 2018), where they play a significant role in the maintenance, amplification, and spread of AIV due to a combination of high poultry density, mixing of poultry species and sources, and generally low biosecurity. Straddling the human-avian interface, they are also a common source of zoonotic infections (Turner et al. 2017; Artois et al. 2017; Junru Ma et al. 2019). Grappling with the role of LPMs, and possible countermeasures for their role in AIV transmission, has occupied substantial attention over the past 5 years.

Poultry housed in LPMs display higher levels of AIV positivity than their counterparts on farms (Junru Ma et al. 2019). The close proximity of multiple poultry species (e.g. chickens, ducks, and geese) allows numerous viral subtypes to spread freely and often asymptomatically, swapping internal genes and rapidly generating novel variants (S. Su et al. 2017; Sung-su Youk et al. 2020). Analyses of poultry workers regularly find notably high seroprevalence of various AIV subtypes (Peiris et al. 2016; Chaudhry et al. 2020; R. Zhang et al. 2021) and zoonotic cases throughout the population are commonly traced back to exposure at LPMs (Lin et al. 2017; Junru Ma et al. 2019; Peiris et al. 2016). Many solutions have been proposed to reduce the risk of AIV maintenance and transmission at LPMs, with varying degrees of corresponding economic impact. Ma *et al.* examined the effects of LPM closure on H7N9 spread in Jiangsu Province during the 2013-2017 outbreaks and concluded that 3-4 weeks of closure were necessary to control the spread of infection (Junru Ma et al. 2019). Wang and colleagues, studying the same string of outbreaks, suggested encouraging the movement of poultry production away from LPMs and regular sero-surveillance of high-risk market workers (D. Wang et al. 2016). Unsurprisingly, measures often meet substantial resistance from LPM workers who rely on the markets for income – closures in China during the 2013 H7N9 outbreak alone, for instance, cost the poultry industry an estimated 8 billion USD (Peiris et al. 2016). There has been a recent push to validate LPM outbreak control methods that remain effective while incurring lower costs on the local economy.

These include the introduction of rest/cleaning days, banning of overnight poultry holding, and separation of chicken and duck populations (Peiris et al. 2016). However, even these less costly alternatives remain unpopular (X. Lei et al. 2019), suggesting that additional education and economic incentives would be required to raise compliance with any new safety guidelines (Lin et al. 2017).

LPMs and other sites of high poultry density facilitate AIV maintenance and reassortment, but virus introduction and interregional spread require vectors capable of inter-regional travel. Human activity is a common source of AIV spread (Delabouglise et al. 2017; Artois et al. 2018). Lu *et al.* conducted phylogeographic analyses to determine regional routes of transmission, finding that human transport (e.g. freight movement of poultry) is primarily responsible for the westward spread of new AIV strains across China (Lu Lu, Leigh Brown, and Lycett 2017). Examining the North American HPAI H5N2 outbreak of 2014-2015, Hubbard *et al.* found unrestricted transport and other lapses in biosecurity measures to be the most likely factors responsible for the rapid spread of the virus (Hubbard et al. 2017). Similarly, Hicks *et al.* conducted viral phylodynamic and ecological modelling of the same outbreak and concluded that multiple intercontinental introductions of the virus were not required for the observed trends. Instead, the outbreak was likely self-sustaining, with existing biosecurity measures sufficient to prevent continuous outside introductions but ineffective against local transmission via unrestricted movement between densely populated commercial poultry farms (Hicks et al. 2020). Human travel has also been confirmed as a major contributor to spread of AIV between commercial farms with generally higher biosecurity and surveillance than smaller-scale farms and LPMs (Young et al. 2017). Kouam *et al.* examined two HPAI H5 outbreaks in Cameroon (2006 and 2016-2017), finding the primary factors in transmission to be unrestricted movement between poultry and egg farms, poor biosecurity measures on these farms, and the presence of LPMs (Kouam, Tchouankui, and Ngapagna 2019). Intercontinental human transport of AIV also occurs; illegal poultry imports are increasing, and HPAI H5Nx viruses have been isolated from raw chicken products brought to Japan by plane passengers (Shibata et al. 2018).

While human activity contributes to regional AIV spread, the primary vector of transboundary AIV spread is wild migratory birds (The Global Consortium for H5N9 and Related Influenza Viruses 2016; Lycett et al. 2020). Influenza prevalence in migratory birds, and the subsequent transmission patterns caused by their movements, is a massive and growing field of research spurred by recent developments in surveillance capabilities and computational modelling (discussed in more detail below). Migrating bird populations can exhibit high AIV seroprevalence (Endo and Nishiura 2018) and often have high natural resistance to AIV, allowing them to carry influenza undetected (W. S. Liang et

al. 2020; Verhagen, Fouchier, and Lewis 2021; Pantin-Jackwood et al. 2016). Humphreys *et al.*, who used satellite-marked blue winged teal (*Spatula discors*) to model North American migrating duck populations, found that their migratory pathways correlated with increased risk of local AIV outbreaks (Humphreys et al. 2020). Resting and wintering sites along migration routes contain multiple different species in close contact, facilitating viral spread and evolution (Mine et al. 2018; Dalby and Iqbal 2015). Where major migratory flyways intersect with densely-populated land regions, such as the Nile River valley, transmission to domestic birds can be common, rapid, and extremely difficult to detect or prevent (Young et al. 2017). The flyways of different species often overlap in breeding grounds, allowing mixing and transmission of viruses across continents. Mine *et al.*, for instance, conducted a phylogeographic analysis of a 2017-2018 HPAI H5N6 outbreak in Asia and Europe and found evidence for a common ancestor in a mixed breeding ground in Siberia, where three major migratory flyways are known to converge (Mine et al. 2018).

Beringia is another area of significant species overlap, hosting large numbers of migratory birds and facilitating AIV spread between Asia and North America via the East Asia-Australia, Pacific Americas, and Mississippi Americas Flyways (Chaudhry et al. 2020; Mine et al. 2018). Beringia facilitates the highest levels of Asia-to-North-America wildlife movements of any migratory hotspot (Morin et al. 2018), and it was from this area that the North American HPAI H5N2 outbreak likely originated (D.-H. Lee et al. 2015). Wide-ranging species, such as emperor geese (*Anser canagicus*), collect in the Beringia and have shown high seroprevalence of diverse AIV subtypes (Ramey et al. 2019). Such hotspots host a complex pattern of influenza propagation, with some species primarily responsible for amplifying AIV infections (particularly LPAI viruses) in local wintering grounds (S. Yin et al. 2017), while others with longer infectious periods serve to transmit novel viruses to distant locations (Xueying Li, Xu, and Shaman 2019). Some IAVs shed by migratory birds have been found to persist in surface water for >7 months, potentially serving as a long-term reservoir during overwintering periods (Ramey et al. 2020).

Interactions between domestic poultry and migratory birds can be common and difficult to avoid in developing countries, and migration flyways often cover areas of high poultry density (Lu Lu, Leigh Brown, and Lycett 2017). Kwon *et al.* observed that the 2014-2016 Korean H5N8 clade 2.3.4.4 outbreak began with multiple introductions of the virus from wild waterfowl, with most Korean domestic duck farms being located near the wintering sites of migratory birds (J. H. Kwon et al. 2020), and outbreaks in Southeast China are commonly linked to the movement of birds along the East Asian-Australasian Flyway (Lu Lu, Leigh Brown, and Lycett 2017). Anthropogenic factors also commonly increase the risk of these interactions. Before the 2005 H5N1 outbreak at Qinghai Lake, for instance,

the local population had established goose farms to provide food for workers constructing a railway nearby. These farms mixed domestic birds with captured wild geese, which then spread the virus throughout the country; H5N1 genetically traced to this emergence was later reported in at least 61 countries (Canavan 2019). Growing urban spread and habitat destruction will likely increase contact between wild and domestic bird populations (Gilbert et al. 2017; Morin et al. 2018), and thus the surveillance of AIV strains present in migratory hotspots has become an especially critical field of research since 2015. Current sampling strategies tend to be opportunistic rather than active, "uneven in host, time, and geography" (Morin et al. 2018), and biased toward easily accessible migratory populations, severely limiting our ability to monitor the emergence and spread of potentially dangerous novel AIV strains (Verhagen, Fouchier, and Lewis 2021).

Cross-transmission – the spread of AIV back to wild birds from domestic poultry – also plays an important role in the maintenance of AIV and generation of viral diversity (Bahl et al. 2016). Zhang *et al.* point out that wild birds harbour greater diversity in HA/NA subtypes, but not in internal gene segments, compared to domestic poultry, suggesting that cross-transmission may be more common than previously thought (T. Zhang et al. 2019). Though far smaller than most LPMs, unregulated backyard farms are common across the world and can also form environments conducive to AIV swapping with wildlife. In Scotland, for instance, such farms are not reportable if they contain <50 birds, yet multiple poultry species are regularly intermixed in environments that are readily accessible to migratory birds (Correia-Gomes and Sparks 2020). Backyard poultry farms almost universally lack sufficient biosecurity measures (Al-Ebshahy and Abotaleb 2019; Derksen et al. 2018; Chaudhry et al. 2018), suggesting a widespread need for greater education and access to personal protective equipment (PPE) and disinfection tools (Young et al. 2017).

Non-migratory wild birds can also contribute to community spread of AIV, either directly or by linking migratory birds to domestic poultry populations (W. S. Liang et al. 2020). Wild crows in Bangladesh, for instance, display extremely high seroprevalence for H5N1 and often live on offal from live bird markets (Hassan et al. 2017). Employing a modern approach based on satellite imagery combined with phylogeographics, Liang *et al.* studied the ecological factors behind clade 2.3.4.4 HPAI viruses in Taiwanese poultry farms. They observed links to high poultry farm density and heterogeneity (presence of multiple poultry species facilitating viral evolution) and an interesting connection to high percentages of cropping land coverage – this may allow greater mixing of non-migratory birds with their migratory counterparts, increasing the likelihood of transmission to domestic poultry (W. S. Liang et al. 2020).

Wildlife and Natural Reservoirs

Wild waterbirds of the orders Anseriformes (e.g. ducks, geese, and swans) and Charadriiformes (e.g. gulls, terns, and shorebirds) are considered the natural reservoir of LPAI AIV, and their migratory patterns and interactions with domestic poultry form the backbone of most established AIV transmission networks worldwide (Verhagen, Fouchier, and Lewis 2021; Marché, van den Berg, and Lambrecht 2018). In addition to elucidating these transmission networks as discussed above, surveillance and monitoring studies are also critical for identifying unknown or understudied AIV hosts and potential reservoirs that may influence viral spread through migratory pathways. In eastern Mexico, for instance, where the three main North American migration flyways converge, Cerda-Armijo *et al.* detected via RT-PCR >60% AIV prevalence in land birds (e.g. cardinals, cowbirds, and blackbirds) and ~27% prevalence in aquatic birds, a finding that emphasizes the importance of monitoring not just waterfowl but also the intermediate species with which these reservoirs may interact (Cerda-Armijo et al. 2020).

Small-scale studies, focusing on specific species or locations, can reveal unnoticed patterns in AIV prevalence and diversity with potential relevance to larger populations. Lee *et al.*, for instance, found in a study of Svalbard waterfowl that Charadriiformes harbour dominant influenza subtypes different from those in Anseriformes, suggesting that these groups may serve as reservoirs for separate pools of AIV diversity and thereby facilitate reassortment events at site where they intermingle (M. M. Lee et al. 2020). A coincident analysis of African penguins (*Spheniscus demersus*) on Halifax Island, Namibia, determined the cause of a recent die-off to be HPAI H5N8 clade 2.3.4.4b, a subtype that had previously caused outbreaks in South Africa in 2017 (Molini et al. 2020). The overlap of migratory flyways with sea mammal habitats can also result in dangerous AIV reassortment and adaptation. Outbreaks of various avian-origin influenza subtypes have been reported in marine mammals, with effects ranging from subclinical to mass die-offs (Fereidouni et al. 2016). Amino acid changes in the HA protein have been found to occur rapidly when avian influenza jumps from birds to seals, for instance (Bodewes et al. 2016). Venkatesh *et al.* reported the first full-genome sequencing of an AIV (H3N8, in this case) isolated from a grey seal. Protein alignment revealed unusual residue changes including the rare D701N PB2 mutation, known to promote mammalian adaptation in avian viruses (Venkatesh et al. 2020). In another recent small-scale study, Guan *et al.* found that gull-origin H10N7 virus is capable of aerosol transmission between ferrets without prior adaptation (M. Guan et al. 2019).

Anthropogenic factors can also contribute to changes in the behaviour of wild avian species, increasing the risk of interactions between waterfowl reservoirs and domestic poultry. The American white ibis (*Eudocimus albus*) has become increasingly urbanized due to habitat destruction and typically nest in dense, species-diverse colonies. Vulnerable to AIV and exhibiting high seroprevalence, they are another potential wild influenza reservoir (Bahnson et al. 2020). In China, rice paddy coverage has expanded rapidly to meet the needs of food security, increasing contact between domestic and wild migratory ducks (Morin et al. 2018). Paddies are often planted next to natural wetlands for convenience and may themselves serve as artificial habitats for many waterfowl, altering migratory pathways and influencing the long-distance spread of dangerous viruses like HPAI H5Nx clade 2.3.4.4 (Gilbert et al. 2017). Further research will be required to measure the impact of human development on influenza transmission patterns, particularly as an increasing population and climate change continue to complicate the issue. Land clearance is never without consequence on local wildlife populations, and sudden changes to ecological pathways can have unexpected and dramatic consequences on the pattern of interactions between domestic and wild animals.

Epidemiology of Swine Influenza

One of the main threats posed by swine within influenza epidemiology is a result of their unique respiratory cell biology. Avian influenza viruses primarily bind to α-2,3-sialic acid (SA) residues present on avian respiratory and intestinal cells, while human viruses prefer the α-2,6-SA residues that are more common in the human host. Swine present both types of SA throughout their respiratory tracts, making them an effective intermediate host or “mixing vessel” capable of hosting widely varying influenza subtypes and facilitating reassortment events (Hicks et al. 2020; M. S. Park et al. 2020). This flexibility was exemplified in the 1998 emergence of triple reassortant gene (TRIG) swine viruses comprising a novel mix of human-origin HA/NA and avian- and swine-origin internal genes (Vincent et al. 2017) that subsequently spread widely. In 2009, reassortment between a North American TRIG strain and a Eurasian avian-like H1N1 strain produced the H1N1 pandemic strain (H1N1pdm09). This strain, undetected in pigs before emerging in humans, spread zoonotically and transmitted rapidly from human-to-human, causing substantial morbidity and mortality worldwide (Mena et al. 2016).

The 2009 H1N1 pandemic was a dramatic example of the dangers associated with unmonitored influenza maintenance within swine populations, and research efforts have escalated over the past 10 years to increase our surveillance and monitoring capabilities to track ongoing viral spread and give

forewarning for dangerous reassortment events (Vincent et al. 2017). Current efforts include phylogenetic and seroprevalence studies of viruses circulating in domestic swine holdings, examination of zoonosis and reverse zoonosis along the human-swine interface, and research into swine influenza transmission and the broader role of swine within the avian-swine-human viral network.

Current Global Situation

H1N1pdm09 remains one of the most important human seasonal influenza strains (J. G. Yoon et al. 2021) Representative studies have shown that H1N1 strains, including H1N1pdm09, are also one of the most important viruses currently circulating in swine populations worldwide, along with H1N2 and H3N2 (Cador, Rose, et al. 2016; P. Zhao et al. 2019; Henritzi et al. 2020). Wong *et al.* examined the phylogenetics of swine influenza virus (SIV) isolated from two swine populations in Australia between 2012 and 2016, finding gene segments from human-origin viruses circulating widely. These pandemic-adjacent strains – including H1N1/1977-like, H1N1/1995-like, H3N2/1968-like, and H1N1pdm09 – are likely to have been circulating for decades as a result of reverse zoonosis and transmission to naïve animals introduced into the swine farming system (Wong et al. 2018). Similar strains have been identified in pig farms in Africa (Ayim-Akonor et al. 2020) and Latin America (Mena et al. 2016). In the latter case, Mena *et al.* noted that the European-lineage H1N1 virus that contributed to the emergence of H1N1pdm09 in Mexico was likely to have been introduced via imported swine on at least two separate occasions. These introductions may have occurred as far back as the 1990s, highlighting the long-term risk posed by even relatively low levels of international livestock movement without adequate surveillance (Mena et al. 2016).

Recently, Henritzi *et al.* conducted a large-scale study of SIV presence in European swine holdings, combining phylogenetics with passive surveillance of nearly 2500 separate holdings to estimate SIV diversity and prevalence since the last major European surveillance programme (ESNIP3) ended in 2013 (Henritzi et al. 2020). Their results showed a substantial increase in the overall prevalence of SIV-infected farms since then (from ~31% to ~57%), with avian-origin H1N1 as the dominant strain and H1N2, H3N2, and H1N1pdm09 strains also in circulation. Reverse zoonosis and subsequent reassortment (discussed in detail below) was a significant factor increasing genetic diversity. Of particular concern was the finding that several of the observed SIV reassortants seemed to have acquired resistance to the human antiviral MxA protein, a trait associated with the pandemic influenza viruses of 1918 and 2009 (Henritzi et al. 2020). In Egypt, along with avian-origin H5N1 and H9N2,

H1N1pdm09 also remains endemic in swine despite mass culling of pigs in the aftermath of the 2009 pandemic (Gomaa et al. 2018). Sun *et al.* recently identified a reassortant, circulating in China since 2016, which contains H1N1pdm09 and triple-reassortant-derived internal genes and exhibits several concerning properties – namely, binding preference to human-type receptors, efficient replication in human airway epithelial cells, and low cross-reactivity with current vaccine strains (H. Sun et al. 2020). Human-to-human transmission has not been reported (Centers for Disease Control and Prevention 2020), but experimental airborne transmission is possible between ferrets (H. H. Sun et al. 2020).

Meanwhile, zoonotic transmission of SIV remains a significant concern due to the biology of swine that contributes to the mammalian adaptation of avian-origin viruses. Close contact between humans and swine is common throughout the world in various industrial and recreational contexts including swine farming, live animal markets, and livestock shows. Small-scale pig farms are very often unregulated and allow unnoticed, unrestricted spread of SIV between local holdings (Mateus-Anzola et al. 2019). Nelson *et al.*, studying factors behind a recent shift from H3N2 to H1N2 in reported swine-to-human zoonoses, describe a single 2018 early-season national jackpot swine show that played an important role in "seeding" a particular human-origin H1 subtype (H1δ-2) that subsequently spread nationwide (Nelson et al. 2020). Such livestock shows are common throughout the USA and rarely enforce adequate biosecurity, providing optimal conditions for SIV to transmit between pigs and humans.

Modes and Routes of Transmission

SIV strains spread rapidly within densely-populated swine farms and can be transferred between holdings via livestock movement, reverse zoonosis, and introduction by wild birds (M. S. Park et al. 2020). Cador *et al.* noted that in farrow-to-finish farms, where all steps from swine breeding through growth to market weight are conducted in the same confinement operation, particularly great care must be taken to avoid the spread of H1N1, H1N2, H3N2, and other common SIV. Maternally-derived antibodies (MDA) temporarily reduce susceptibility to SIV in piglets but do not fully prevent transmission, masking infections and potentially leading to silent spread in the first weeks of life (Cador, Hervé, et al. 2016a). Fitzgerald *et al.* examined correlations between production flow and SIV seroprevalence in similar farrow-to-finish farms, concluding that some common policies – e.g. mixing of pig groups and reintroduction of previously hospitalized piglets – contribute to SIV circulation (Fitzgerald et al. 2020). The practice of using nurse sows, wherein a single sow may feed multiple litters of piglets, has also been associated with increased risk of SIV transmission. Piglets are at particular risk

of influenza infection, and infected animals have been found to shed high titres of SIV onto the udder skin of nurse sows. This in turn can transmit the virus to other batches of uninfected piglets, where clinical signs of infection may be masked by the presence of MDA (Garrido-Mantilla, Culhane, and Torremorell 2020). Infected piglets, which are often transferred to new holdings after weaning, can easily spread SIV between farms, although adequate sow vaccination and negative gilt SIV status at entry were associated with reductions in these risks (Fabian Orlando Chamba Pardo et al. 2018).

Surveillance of SIV during long-distance swine movement has expanded since 2009 but remains imbalanced and highly limited compared to AIV surveillance, particularly in developing countries (Mena et al. 2016). Phylogenetic analyses demonstrate that the global live swine trade is highly correlated with epidemiological patterns of SIV, with North America and Europe primarily serving as exporters to Asian countries (Nelson, Viboud, et al. 2015). As noted above, however, trade with developing countries is often subject to less extensive biosecurity and can result in the transmission of avian-, swine-, and human-origin viruses with unpredictable consequences (Mena et al. 2016).

Meanwhile, although zoonotic transmission from swine to humans occupies a higher research share, reverse zoonotic transmission from humans to swine has a significant role in the generation of viral diversity in pig populations. By monitoring SIV introductions in the USA between 2009 and 2014, Nelson *et al.* noted that H1N1 segments associated with the H1N1pdm09 strain had largely disappeared from the US swine population by 2013 before re-emerging via multiple human-to-swine transmission incidents in one of the largest-scale reverse zoonotic events ever recorded (Nelson, Stratton, et al. 2015). Reverse zoonosis to swine can rapidly result in reassortants with properties well-suited to mammalian transmission - for example, Rajão *et al.* identified a novel human-like H3N1 virus in swine in the USA composed of human seasonal H3N2 HA, classical swine H1N1 NA, and internal genes derived from H1N1pdm09 (Rajão et al. 2015). This virus seems to be well-adapted for airborne transmission and was noted to have spread from its index case in Missouri to another state without known epidemiological links, suggesting concerning gaps in the SIV surveillance network (Rajão et al. 2015).

Finally, wild birds can facilitate long-distance transmission of SIV by interacting either directly with swine or indirectly through domestic poultry populations (Joseph et al. 2017; M. S. Park et al. 2020). Studies of live poultry market workers in Guangdong Province, China, have found roughly equal susceptibility to both avian and swine influenza viruses, the latter of which may be carried by some bird species or by swine present in the market (J. Chen et al. 2015). As human development continues

to expand agriculture into migratory flyways and other areas of high waterfowl density, interactions between domestic swine and wild birds may become more common, introducing more genetic diversity into existing swine influenza networks and raising the risk of novel reassortments producing dangerous hybrid viruses (Lycett, Duchatel, and Digard 2019; M. S. Park et al. 2020; Lloren et al. 2017; Wong et al. 2018).

Epidemiology of Equine Influenza

As equids are not a substantial component of international meat production and trade, and zoonotic transmission is extremely rare, equine influenza virus (EIV) is often neglected in virological and epidemiological research. Equine outbreaks are common, however, and can cause substantial economic disruption. In 2007, for instance, an EIV strain was introduced to Australia via thoroughbred horses from Japan and escaped quarantine due to lax biosecurity measures, spreading rapidly despite widespread vaccination and causing a 5% mortality rate in infected horses (Sack et al. 2019). The total cost of the outbreak, including measures taken to shore up biosecurity and surveillance networks, was nearly 1 billion USD.

As with SIV, recreation is a significant and understudied component of the human-animal interface that can facilitate transmission of EIV between horse holdings and across country borders (Spence et al. 2017). Many recent studies of equine influenza have focused on South America, where occasional outbreaks have been recorded for decades. Perglione *et al.* examined two recent outbreaks, in 2012 and 2018, of EIV in Argentina and surrounding countries. In the first outbreak, clinical signs of respiratory disease were noticed in racing and training horses in Uruguay before spreading to Argentina. Sequence analysis confirmed that the cause was H3N8, closely related to viruses isolated in the USA in 2012. An identical virus was also isolated from vaccinated horses in Dubai that had been imported from Uruguay (Perglione et al. 2016). The 2018 outbreak, primarily restricted to Argentina, was associated with the same virus strain (H3N8 Florida clade 1), but with several amino acid substitutions that facilitated immune escape despite regular vaccination of racing horses. Equine competitions and movement of horses were not restricted during the outbreak, likely leading to wider spread (Olguin-Perglione et al. 2020). Bravo-Vasquez *et al.* examined the epidemiology of equine influenza in Chile, where equine-like internal genes have been identified in avian influenza virus isolates. H3N8 remains endemic in the country and shows hallmarks of adaption to avian hosts, permitting maintenance and transmission in wild and domestic birds (Bravo-Vasquez et al. 2020).

In Mongolia epizootic EIV outbreaks with relatively high mortality rates (~20-30%) are common among the country's large domestic horse population (Sack et al. 2019). Soilemetzidou *et al.* studied the extent to which these outbreaks are shared with Mongolia's two wild equid species, the Asiatic wild ass and the Przewalski horse. They found seroprevalence of numerous EIV subtypes including H7N7, which had been thought extinct in the region. These wild horses may therefore represent an understudied ecological reservoir for EIV (Soilemetzidou et al. 2020).

Continuing outbreaks of EIV, and the ability of antigenic drift to rapidly generate vaccine-escape variants, highlight the need to continue and expand existing EIV surveillance programs and enforce biosecurity measures currently in place for preventing international EIV transmission. Additionally, historical and serological evidence suggests that EIV strains have the potential to develop human-adaptive traits unexpectedly, making it a potential epidemic threat (Sack et al. 2019). Horses display both α-2,3- and α-2,6-SA residues in their respiratory tracts, potentially facilitating antigenic drift toward human-adapted genotypes (P. Zhou et al. 2019).

Influenza in Companion Animals

Domestic cats and dogs are potential reservoirs for influenza mammalian adaptation due to their proximity with humans and, often, infected wildlife. Thought for many years to be naturally resistant to influenza viruses, companion animals have more recently been found to be susceptible to a wide range of influenza subtypes, including avian-origin viruses. As companion animals are an uncommon source of zoonotic infections, research into the epidemiology of associated veterinary outbreaks is rare. Recent studies suggest caution and continual monitoring, however in 2016, for example, transmission of H7N2 from a cat to a veterinarian was recorded in a New York animal shelter (Belser et al. 2017). In South Korea, during the 2014-2016 HPAI H5 outbreak, several cats with access to migratory birds as prey were found to be infected with H5N6 similar to isolates from nearby poultry farms (K. Lee et al. 2018). Meanwhile, Ibrahim *et al.*, conducting seroprevalence surveys of domestic cat sera during and after the 2009 H1N1 pandemic, found a high level of feline exposure to human influenza virus infection that correlated with IAV prevalence in the human population (Ibrahim et al. 2016).

Canines are susceptible to infection by equine-origin H3N8, and cases of avian-origin H3N2 have been reported in the United States and Eastern Asia (He, Li, Zhu, et al. 2019). H3N2 seems to be adapting to dogs as a host and can cause viral shedding for more than 21 days during infection, stymying

outbreak control protocols based on the shorter infectious period of H3N8 (Newbury et al. 2016). Canines may also transmit this virus to horses via close contact (P. Zhou et al. 2019). Dogs, as with horses, display both α-2,3- and α-2,6-SA in their respiratory tracts, potentially raising the risk of human-adaptive mutations in canine viruses (P. Zhou et al. 2019). Recent data from the National Institutes of Allergy and Infectious Diseases CEIRS network show that canine H3 influenza A viruses are capable of replication in human primary nasal/bronchial epithelial cells and experimental transmission in mammalian models while exhibiting sensitivity to existing antiviral drugs (Martinez-Sobrido et al. 2020).

Zoonosis and Reverse Zoonosis

The primary factor associated with zoonotic transmission of veterinary influenza is close contact between humans and domestic animals. Such contact is often unregulated and carried out with minimal biosecurity precautions (Derksen et al. 2018), and the number and size of high-density animal farms is rapidly increasing across the world (Hautefeuille, Dauphin, and Peyre 2020; Shuo Liu et al. 2020). In recent years, reverse zoonosis – the transmission of virus from humans back to animals – has also received increasing attention for its role in generating viral diversity and mammalian adaptations.

Zoonosis of avian influenza is especially common in countries with long-standing systems of LPMs, and while AIV often jump first to swine and then to humans, direct human transmission is possible without requiring long periods of mammalian adaptation (S. Su et al. 2015). Egypt has recently been the epicentre of a large number of human H5N1 infections, with more confirmed cases of zoonotic avian H5N1 than any other country (Young et al. 2018). Young *et al.*, tracking key amino acid substitutions that might facilitate viral adaptation to human hosts, observed such substitutions in 94.5% of Egyptian viral isolates with significant clustering in the densely populated Alexandria and Beheira Governorates (Young et al. 2018). Backyard chickens are a constant reservoir of AIV in Egypt, and incomplete vaccination strategies can promote the emergence of novel subtypes and escape variants (Al-Ebshahy and Abotaleb 2019). H5Nx clade 2.3.4.4 is a growing concern in many developing countries, including Latin America and the Caribbean where avian influenza is one of the most frequently reported emerging zoonoses (Maxwell et al. 2017).

HPAI H5Nx viruses deserve careful attention due to their high case fatality rate. Worldwide, these viruses have caused at least 861 human cases and 455 deaths (Salvador et al. 2020). However, zoonotic

cases of H5N1 have decreased drastically since 2016, while H5Nx clade 2.3.4.4 viruses in general do not appear amenable to airborne transmission or replication in mammals (Sutton 2018). Exceptions seem to exist, however – clade 2.3.4.4 H5N6, for instance, has the potential to cause severe human disease and is currently spreading rapidly from China via asymptomatic migratory ducks (Lycett, Duchatel, and Digard 2019; W. S. Liang et al. 2020; Bi et al. 2016). Seven human cases of HPAI H5N8 were recently observed in Russian poultry workers, with minimal-to-no symptoms (Adlhoch et al. 2021). Molecular epidemiology and active surveillance will play an important role in continually monitoring such viruses for the acquisition of traits promoting zoonotic transmission. It is also critical to direct research toward understudied potential influenza reservoirs in the wild – little brown bats, for instance, are widely distributed in North America and have been found to express both α2,3- and α2,6-SA in their respiratory and gastrointestinal systems, making them potentially amenable to coinfection with avian, swine, and/or human strains (Chothe et al. 2017).

Unlike most H5Nx viruses, LPAI H7N9 does show signs of airborne transmission, and Asian-lineage H7N9 viruses are recognized by the CDC as the highest potential pandemic risk among AIV (Sutton 2018). Since the emergence of an HPAI variant during the 2016-2017 fifth wave of the China H7N9 outbreak, both versions remain endemic in Chinese poultry. More *et al.* noted that H5 and LPAI H7 viruses circulate in European migratory birds, while potentially zoonotic H9N2 is common in much of the Middle East, Asia, and north Africa (More et al. 2017)

Swine, meanwhile, are well-known to act as an intermediate host that is amenable to infection with a wide array of avian-, swine-, and human-origin influenza viruses, promoting reassortment and mammalian adaptation. As noted above, reverse zoonosis has a critical and understudied role in the generation and maintenance of novel SIV reassortments worldwide (Henritzi et al. 2020). In a 2020 study of zoonotic infections resulting from livestock shows in the USA, Nelson *et al.* found that 99% of the variant viruses they identified in humans had reassortant genotypes that contained H1N1pdm09 internal genes segments originally introduced into swine from humans (Nelson et al. 2020). These results are in line with similar data from Australia, where segments from decades-old pandemic influenza viruses have been found circulating widely (Wong et al. 2018). H3 swine viruses remain a particular concern. Zoonotic infections in the USA have recently shifted from H3N2 to H1N2 (Nelson et al. 2020), but H3 remains endemic not only in North America but in regions of Africa (Ayim-Akonor et al. 2020), Asia (He, Li, Zhu, et al. 2019), and Europe (Henritzi et al. 2020). Henritzi *et al.* noted that in Europe, new H3 viruses began circulating in areas with H3-naïve swine populations but then spread to areas with constant H3 exposure, indicating lack of cross-protection (Henritzi et al. 2020).

Particularly virulent H3 strains have been identified in birds, swine, horses, dogs, and many other species, and more research is required to track their spread and identify potential unknown reservoirs in the environment (P. Zhao et al. 2019; Newbury et al. 2016; Bravo-Vasquez et al. 2020; Venkatesh et al. 2020). Interestingly, human coinfections with seasonal H3N2 and avian H7N9 have been reported, raising the possibility that humans may act as a mixing host for reassortment under certain circumstances (S. Su et al. 2017).

Increased surveillance and research are also needed for the recently-discovered influenza D virus (IDV), an important component of the bovine respiratory disease complex. First identified in North American swine in 2011, but now associated primarily with cattle, IDV is not known to be pathogenic in humans. However, high IDV seroprevalence (>90%) has been found in some bovine workers (White et al. 2016), and evidence of airborne transmission has been observed from experimental infections (Salem et al. 2019). IDV also displays receptor-binding preferences similar to those of human influenza viruses (Horimoto et al. 2016). IDV is highly prevalent across the world (Horimoto et al. 2016), but most of its basic properties – pathogenesis, tropism, transmission routes, etc. – remain critically under-explored (Salem et al. 2019; Horimoto et al. 2016), particularly in developing countries (Fusade-Boyer et al. 2020). IDV is likely to have circulated worldwide in swine and cattle long before its discovery, emphasizing the need for enhanced surveillance capabilities encompassing species not commonly associated with influenza infections.

Zoonosis and reverse zoonosis are important pathways by which viral diversity is generated (Chastagner, Enouf, et al. 2019), but large-scale surveys and representative studies have shown that these risks are often underestimated by farmers and workers in close contact with domestic animals. In a questionnaire-based study, Venkat *et al.* found that veterinarians in Arizona, USA, often fail to report cases of zoonotic infections, and only 37% of those surveyed regularly wore PPE when handling clinically ill animals (Venkat, Yaglom, and Adams 2019). In India, which is considered to have a high risk for the emergence of new diseases, a survey of poultry farmers showed generally low knowledge of zoonosis and regular practice of unsafe farming/animal handling methods (Hundal et al. 2016). Biosecurity regulations within the swine industry as a whole are often lax, with vaccination and sick leave policies rarely enforced (Nelson, Stratton, et al. 2015). The condition of both animals and humans must be considered when designing policies to curb zoonosis/reverse zoonosis, and widespread improvements in education, regulation, and outreach will be necessary to increase control of this increasingly volatile area of viral generation.

Climate Change and Environmental Epidemiology

Climate change presents a unique and unpredictable challenge for veterinary influenza research that has only grown more urgent over the past 5 years. Warmer climates will have multiple impacts on the maintenance and spread of influenza viruses, and the interactions between these factors may be difficult or impossible to predict beforehand. Colder weather, for instance, has often been found to increase incidences and persistence of various influenza viruses. Studying factors underlying a recent outbreak of HPAI H5N1 in Egypt, Salaheldin *et al.* found that winter weather was significantly correlated with number of reported outbreaks from 2006-2015 (Salaheldin et al. 2018) – similar results have been reported worldwide (Lau et al. 2019; W. S. Liang et al. 2020; Delabouglise et al. 2017). However, the impact of a warmer climate on other factors that promote survival of AIV in the environment (such as elevated pH and low salinity of water reserves) is unclear (Morin et al. 2018), particularly when paired with other variables like wildlife habitat destruction and skyrocketing domestic meat production (Hautefeuille, Dauphin, and Peyre 2020; Shuo Liu et al. 2020). A warming climate has also contributed to a recent surge in wild swine populations worldwide – concurrently, environmental and habitat disturbance may increase the population density of these swine and facilitate SIV maintenance and zoonotic transmission at the human-animal interface (Ruiz-Fons 2015). Overall, the picture painted by recent research on influenza climatology suggests caution and concern.

Long-term studies and recent modelling have suggested that accelerated warming could bring animal species together in new combinations and promote increased pathogen transmission. Epidemiological climate research is needed to narrow down surveillance programmes to areas of greatest risk (Morin et al. 2018). In some cases, an effective outbreak response may even be at cross-purposes with environmental practices. Salvador *et al.* noted that the common bird disposal method of burying can release leachates into the groundwater, while burning contributes to the emission of highly toxic dioxins (Shuo Liu et al. 2020; Salvador et al. 2020). Recommendations to build poultry cages from easily cleanable – but nonbiodegradable – materials like plastic rather than wood or bamboo (Peiris et al. 2016) are another such example. Where a balance exists between outbreak control and environmental preservation, economic and public health dictate that outbreak control must come first, but an increased research focus on sustainable practices and environment-friendly methods of disinfection and disposal would help to ensure that both needs can be met in the long-term (Morin et al. 2018).

More research is urgently needed to model and estimate the impacts of climate change on influenza transmission mechanics as the planet's temperature continues to rise. The impact of climate on

influenza epidemiology is difficult to study and therefore often ignored in favour of more tangible variables. Current surveillance programmes tend to be intermittent and reactive rather than proactive, facilitating unnoticed transmission and diversification of viruses particularly in wild avian populations (Morin et al. 2018). Where changing climatic variables are altering wild population distributions and migratory routes, proactive surveillance will become ever more critical to ensure our ability to respond quickly to novel strains or particularly dangerous reassortants. Partnerships between epidemiologists and climate modelling technologies (discussed in more detail below) will allow us to focus on areas of particular interest and ensure that limited resources go where they are needed most (Morin et al. 2018; Canavan 2019).

Molecular Epidemiology and Computational Modelling

Continual advances in computing power have facilitated corresponding increases in the predictive capacity of computational models for influenza transmission networks. Over the past 5 years, computational modelling has been employed to study the origins and evolution of severe outbreaks, determine variables strongly correlated with viral spread, and estimate the major routes by which influenza is transmitted and maintained. Sun *et al.*, for instance, used the MEGA 6.0 phylogenetics platform to construct an evolutionary tree of H5N1 sequences and evaluate the global spatial risk pattern of this HPAI virus in wild birds (L. Sun et al. 2018), and Perglione *et al.* used similar tactics to track the evolution of South American equine influenza outbreaks (Perglione et al. 2016). Kurscheid *et al.* conducted a social network analysis in Indonesia, mapping the sources and destinations of birds traded in LPMs and then using UCINET analysis software to construct contact networks across the islands of Bali and Lombok (J. Kurscheid et al. 2017). Similar results on the epidemiological importance of informal animal trading were generated by Mateus-Anzola *et al.*, who used the Epimodel R package to estimate the risk of movements between swine farms at a wildlife/livestock interface (Mateus-Anzola et al. 2019). Scott *et al.*, modelling the likelihood of LPAI H7 spread within and between Australian commercial chicken farms, found via partial consequences assessment that shared equipment was the most likely vector for the viral spread (Scott et al. 2018). In a recent study, Holbrook *et al.* employed computationally intensive Bayesian multidimensional scaling to infer the spread of various seasonal influenza subtypes via worldwide air travel (Holbrook et al. 2021).

Dhingra *et al.*, meanwhile, combined governmental data on HPAI H5N1 outbreaks to construct a global suitability model for the virus, finding that viral host distributions – domestic ducks in particular – predicted outbreaks better than land use or climatic variables (Dhingra et al. 2016). Endo & Nishiura

devised a multisite, multispecies susceptible-infectious-susceptible model to examine the role of migration in maintaining AIV along the East Asian-Australian Flyway. They also found that duck species play a particularly important role in the process (Endo and Nishiura 2018). Such studies allow us to focus costly active surveillance strategies on the species most likely to spread and maintain HPAI viruses. Artois & Lai conducted research on HPAI H5N1 and LPAI H7N9 in China, with resulting data from boosted regression tree models showing a strong positive association between outbreaks of the HPAI virus and proximity of LPMs (Artois et al. 2017).

Similar modelling strategies also allow us to test the effectiveness of outbreak control strategies under various computational models. Lewis *et al.* used the North American Animal Disease Spread Model to estimate the efficacy of influenza control methods in Ontario, finding that the stamping out of infected flocks without ring culling resulted in the most efficient outbreak containment. They stress, however, that such modelling studies require information from meat production industries that may be difficult to obtain depending on the region of interest (N. Lewis et al. 2017). Guinat *et al.* conducted additive Bayesian network analyses on the relationship between biosecurity and HPAI H5N8 risk in French poultry farms. They found that the management of vehicles entering and leaving farms was an especially important variable (Guinat et al. 2020).

Modelling studies require not only computing power but also large amounts of high-quality data in order to generate reliable predictions. Most of the foundational data for phylogenetic/geographic studies come from viral genomic sequencing, and next-generation sequencing (NGS) has played a vital role in generating large datasets that show the complete viral diversity in a given region or population. Fusaro *et al.*, for instance, used Illumina MiSeq analysis of AIV samples to study viral evolution and transmission during a 2013 HPAI H7N7 outbreak in Italy, finding that multiple viruses of the same subtype often circulated within single farms (Fusaro et al. 2016). Unfortunately, NGS is generally time-consuming, costly, and dependent on experienced users and stationary instruments. These issues and others have led to the recent development of so-called third-generation sequencing technologies (Verhagen, Fouchier, and Lewis 2021). Though mainly focused on longer reads, these third-generation technologies have also brought increased attention to portability, speed, and ease-of-use – for instance, the recently-developed MinION sequencer is fully portable, thus allowing sample-source data collection (Tyler et al. 2018; Verhagen, Fouchier, and Lewis 2021). Continuing the development of such technologies will be critical for the rapid acquisition of high-quality genomic data used for computational modelling, climate-focused epidemiology, and active surveillance. Great care must also be taken, as Poen *et al.* point out, that sequencing methods be stress-tested and their results checked

for accuracy and repeatability to ensure robustness and compatibility with other experimental approaches (Poen et al. 2020). Once collected, it is equally important that high-quality sequencing data be made available on network databases (e.g. the GISAID EpiFlu database) that allow rapid sharing and coordination of international research.

Epidemiology, Biosecurity, and the Economy

Thorough knowledge of influenza epidemiology goes hand-in-hand with effective biosecurity measures. A strong outbreak response may be effective, but the measures involved may be extremely costly and cause long-term damage to local food production economies (Salvador et al. 2020; Zancanaro et al. 2019). Epidemiological studies allow us to test the efficiency and efficacy of biosecurity tactics, aiming to maximize protection and minimize cost where possible. Surveys and small-scale community investigations can also show us the likelihood that local farms will follow biosecurity measures and the strategies that may be implemented to incentivize compliance.

Lambrou *et al.*, surveying the attitudes and practices toward AIV of farmers in the poultry-dense Chitwan District of Nepal, found that only half of those surveyed believed that AIV was preventable. HPAI and LPAI outbreaks, though common, were rarely reported due to fears of the economic consequences of culling (Lambrou et al. 2020). Similar results came from a survey of LPM workers in Chongqing, where limited biosecurity measures (e.g. rest/cleaning days) were unpopular and nearly half of respondents failed to recognize contact with sick birds or excrement as an infection risk (X. Lei et al. 2019). Farmers and LPM workers are unlikely to pursue safety education or properly implement biosecurity measures unless there is an economic benefit to doing so (Ayim-Akonor et al. 2020). Therefore, it is critical for governments to expand outreach and education programs while providing economic reassurance to farmers who report influenza outbreaks promptly and implement required biosecurity measures (Leibler et al. 2017). Such practices will likely have a feed-forward effect, reducing the overall economic strain of an outbreak at both the local and regional levels (Cui et al. 2019). Salvador *et al.*, for instance, note that smaller poultry depopulation radii are appropriate for control and can minimize poultry loss as long as government agencies are quickly informed of an outbreak (Salvador et al. 2020). It is also important to note that significant increases in biosecurity may be possible at relatively low cost – Wei *et al.*, for instance, demonstrated a design for a simple, low-cost lid that reduced aerosol production from mechanical defeatherers by 57% (Wei et al. 2019). Further research into inexpensive biosecurity methods, or lowering the cost of existing methods, may raise compliance rates (Lambrou et al. 2020).

Biosecurity measures are generally poorly standardized and vary substantially between countries and regions. Bui *et al.* suggest that biosecurity measures must be tightly integrated with epidemiological studies for maximum efficiency, noting that the fundamental differences in transmission dynamics between H5N1 and H7N9 in China make a one-size-fits-all prevention plan unrealistic (Bui et al. 2018). Many countries rely heavily on vaccination to prevent viral spread, but in addition to the risks posed by unexpected antigenic drift/shift, vaccination can conceal asymptomatic infections and facilitate the maintenance and evolution of novel strains (Shuo Liu et al. 2020). Linking vaccination strategies more closely with epidemiological surveillance can decrease this risk by providing up-to-date information on locally circulating influenza strains (Bui et al. 2018).

Strategies for SIV control must take into account the specific economies and cultures in the regions under study. In Bangladesh, for instance, pig farming is stigmatized in certain communities, reducing surveillance capabilities and making implementation of biosecurity measures and health intervention very difficult (Turner et al. 2017). In China, meanwhile, the slow but steady increase in centralization and consolidation of poultry farms has led to higher biosecurity coverage countrywide (Shuo Liu et al. 2020). Biosecurity education and compliance within many LPMs, however, remains low.

Ongoing research

At the University of Minnesota's College of Veterinary Medicine, research is ongoing into biosecurity strategies for the prevention of AIV outbreaks, building a social network model to evaluate the impact of shared system resources (e.g. fomites, materials, equipment, etc.) on viral transmission and methods for controlling this spread. Scientists at Japan's National Institute of Animal Health are currently conducting studies on the molecular epidemiology of HPAI, while at Ghent University, studies within the EU Horizon 2020 project DELTA-FLU explore HPAI maintenance and transmission patterns, LPAI-to-HPAI transition, viral genetic factors associated with reassortment, and other critical topics at the interface between virus, host, and environment. At the Southeast Poultry Research Laboratory, meanwhile, researchers continue to study interrelated aspects of avian influenza host-virus interaction, including pathogenesis, host adaptation, transmission dynamics, and evasion of the host immune response, along with conducting molecular epidemiology studies to discern the origin and evolution of AIV's inter- and intra-hosts.

Also at the University of Minnesota, one ongoing project evaluates the impact of management strategies directed at decreasing SIV transmission during the preweaning period – current results

indicate that such strategies can reduce SIV infections but are not sufficient to wean negative pigs. U of M scientists also study genetic variability of SIV and the role of suckling piglets in maintaining SIV within and between farms at weaning, finding substantial molecular and genetic complexity and co-circulation of multiple strains at the farm and piglet levels. In Europe, as part of the Pig Influenza Genetics, Intervention and Epidemiology (PIGIE) project coordinated by ANSES, researchers are studying the dynamics of SIV in intensive pig herds, where recurrent forms of influenza are becoming more common and may contribute to coinfections and subsequent reassortments. This study involves field-side, *in silico*, and *in vivo* experimentation to probe the determinants of SIV persistence in pig farms. Research at ANSES also focuses generally on factors influencing the epidemiology and evolution of SIV in European farms, including biosecurity measures, vaccination protocols, and herd immunity.

Two new studies at the Friedrich-Loeffler-Institut (FLI) are currently exploring the dynamics and evolution of SIV in Europe and the role of aerosols in transmitting HPAI between poultry holdings. At the FLI's Institute of Diagnostic Virology, research is ongoing into AIV dynamics (including viral, host, and environmental factors) within the DELTA-FLU project. Meanwhile at the UK Animal and Plant Health Agency, the PIGIE program has been developed to define SIV epidemiology and genetic/antigenic evolution in European pig herds and to produce tools for the prevention and control of SIV within these herds. Researchers there are also involved in the Centers of Excellence for Influenza Research and Response (CEIRR) program, which targets emergency response by characterising pandemic-potential IAV strains and their virulence, transmissibility, etc. Involved in CEIRR as well is the Royal Veterinary College at the University of London. This laboratory also conducts studies within the SIV pandemic risk pipeline, the CANARIES program (Consortium of Animal market Networks to Assess Risk of emerging Infectious diseases through Enhanced Surveillance), and the UKRI GCRF One Health Poultry Hub that explores the risk factors associated with expanding poultry production systems and tests new disease control technologies.

Human infections are monitored at the Francis Crick Institute, the Worldwide Influenza Centre (a WHO Collaborating Centre for Reference and Research on Influenza), including the characterization of zoonotic strains, assessment of pandemic risk, and preparation for candidate vaccine viruses. Researchers at the University of Nottingham, UK, meanwhile, are conducting studies of emerging equine RNA viruses in north-western Nigeria, including a seroprevalence study for equine IAV.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of animal influenza epidemiology should be considered priorities for future research:

- *Transmission dynamics of influenza across major interfaces (e.g. wild, domestic, human, and environment), particularly between pigs and humans*
- *Human-animal interface studies at key sites, including social and behavioural sciences*
- *Risk frameworks for pandemic potential and interspecies transmission – pathways and potential mitigations*
- *Computational risk modelling based on epidemiological and surveillance data*
- *Routes and patterns of avian influenza incursion into poultry holdings*
- *Inter- and intra-species transmission dynamics*
- *Transmission of influenza from wild birds into farms*
- *Studies of experimental long-term influenza maintenance within a population*
- *Maintenance and potential for elimination of HPAI in wild birds, including ecology and evolution*
- *Reassortment and continuing spread of H5N8*
- *Epidemiology of the global spread of LPAI in wild birds, including ecology and evolution*
- *Factors that influence the spread/risk of HPAI along new bird migratory routes*
- *Evolutionary origins of influenza A viruses and potential roles played by newly-detected bat flu viruses (e.g. H9, H17, and H18).*
- *Increasing knowledge of global swine influenza circulation, including the roles of novel swine reassortants and their pandemic/zoonotic risk*
- *Prioritization of swine viruses with zoonotic potential for pandemic preparedness*
- *Propagation pathways of swine influenza between herds (e.g. live pig transport, airborne transmission, etc.)*
- *Better understanding of swine influenza persistence in intensive farms to improve control and prevention measures*
- *Dynamics of influenza transmission in various population settings in swine*
- *Strategies for the suppression and local/regional eradication of swine influenza*
- *Epidemiological gap filling in under-surveyed regions*
- *Integration of multiple disciplines (e.g. epidemiology, immunology, ecology, evolution, etc.) under the One Health umbrella*

Virology/Molecular Biology

Influenza viruses are members of the *Orthomyxoviridae* family. Influenza A, the most common and clinically-relevant influenza genus, is an enveloped virus with a single-stranded negative-sense RNA genome. This genome is composed of eight separate linear segments; consequently, co-infection of a single cell allows the production of hybrid viruses with segments from both infecting strains, a process known as reassortment that may lead to "antigenic shift" when it occurs between viruses of different subtypes. Influenza RNA polymerase is also error-prone, a common property of RNA viruses, and influenza viruses accumulate approximately two to eight substitutions per 1000 nucleotides per year (termed "antigenic drift" when occurring within the antigenic proteins/protein domains of the virus) (Lycett, Duchatel, and Digard 2019). Combined, these processes allow influenza to mutate quickly and unexpectedly, with possible gain-of-function mutations including increased virulence/pathogenicity and changes in tissue or host tropism (Lycett, Duchatel, and Digard 2019). During the late 1990s, for instance, human-to-swine influenza transmission resulted in the generation of "triple-reassortant internal gene" viruses combining genetic material from avian-, swine-, and human-origin influenza; later reassortments involving these viruses led to the introduction of H1N1pdm09, the influenza A virus that caused a worldwide zoonotic pandemic in 2009 (Rajão et al. 2015).

As mutations and reassortments continue to generate novel influenza viruses across the world, it is critical to expand our understanding of the molecular determinants of particularly dangerous viral properties (e.g. vaccine escape, increased pathogenicity, antiviral drug resistance, or ability to adapt to mammalian hosts). Mutation studies of influenza genes have identified many individual mutations that are associated with such changes, but critical mutations may remain unknown, and in many cases their combinatorial effects are not well understood. As sequencing and computational analysis technologies continue to advance, our ability to rapidly sequence influenza genomes and perform mutational analyses increases as well. These virology/molecular biology studies can clarify the mechanisms behind viral gains-of-function and allow us to focus surveillance and control efforts on emerging viruses with particularly dangerous genetic characteristics.

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps in animal influenza virology in 2014/15:

- *molecular determinants of virulence*
- *molecular determinants of viral adaptation/restriction to different species/tissues*
- *molecular determinants of viral survival under different conditions*
- *functions of viral proteins*
- *host-virus protein interactions*
- *influence of virus on host gene expression by novel mechanisms, e.g. microRNAs*
- *development of systems to track model viruses* in vivo *in experimental animals*
- *rate of genetic change and factors affecting*
- *effect of mutations at receptor binding site on viral replication*
- *mechanisms of gene reassortment between virus subtypes*

Literature review

Avian Influenza Virus

AIV evolution and antigenic diversification begin within the avian host, where intra-host adaptation and cross-species adaptive mutations facilitate enhanced viral replication, shedding, and transmission. Soda *et al.* applied molecular studies to questions about the evolution of an HPAI H5N8 strain in Japan during the 2014-2015 winter season, finding that a significant increase in chicken pathogenicity could emerge from a single passage in chickens. This increase was attributed to an M374V mutation in the viral NP gene, associated with polymerase activity and leading to rapid growth *in vitro* and *in vivo* (Soda et al. 2019). Recent advances in sequencing technology have allowed in-depth studies of intra-host AIV evolution – research by Leyson *et al.*, for instance, examined HPAI H5N8 genomic diversity in experimentally-infected mallards and chickens to clarify observed differences in mortality and transmission dynamics between species (Leyson et al. 2019).

Mutations that enhance transmission, shedding, replication, and other survival-related factors can lead to rapid dissemination of adapted strains within poultry populations. Long *et al.* studied the adaptations that initially allowed HPAI H5N1 to transmit from wild birds to gallinaceous poultry –

namely altered receptor binding, increased pH of HA fusion, and deletions of the NA stalk (Long, Benfield, and Barclay 2015). DeJesus *et al.*, meanwhile, studied adaptation of HPAI H5N2 clade 2.3.4.4 compared to index virus, finding a complex pattern of mutations that reduced infectious dose thresholds and enhanced contact transmission in some isolates (DeJesus et al. 2016). Further study of this evolutionary adaptive pathway revealed specific mutations in this virus's HA, NA, NP, and PB1 genes that have contributed to viral fitness in chickens (S.-S. Youk et al. 2021). These include mutations in HA (e.g. M66I, S141P, and L322Q) and NA (E368K and S416G) that contribute to the balance between these proteins' functions that is critical for viral fitness (Du et al. 2020), and mutations like PB2-L386V and -V649I that have been associated with viral polymerase adaption to lower temperatures.

Many recent molecular and phylogenetic analyses have focused on the HPAI H5Nx strains that have spread rapidly worldwide over the past five years. Chen *et al.*, studying virulence determinants within the PB2 viral RNA polymerase subunit, discovered a polymorphic site at PB2-283M, where novel mutations began appearing after 2005. PB2-M283L, in particular, has been isolated from mammalian hosts and was found to increase viral replication in mammalian cells (S. Chen et al. 2020). Studies in the earlier H5N1 clade 2.2.1 identified multiple mutations in the PB1, PB2, PA, and NP genes that worked cooperatively with a PB2-E627K mutant known to contribute to human adaptation. These polymorphisms, including the D175N, T182I, K198R, and K214R mutations of PB1 that modify the vRNA promoter-binding β-ribbon region, increased viral growth in human airway epithelial cells and mouse lungs (Arai et al. 2016). Relatedly, the full-length PB1-F2 accessory protein has been found to cause longer viral shedding periods in chickens, but it is often truncated in swine and human viruses, suggesting mammalian adaptations for increased protein stability and pathogenicity (James et al. 2019). In the HA protein, the K193T mutation has been shown to increase the viral binding preference of a ferret-transmissible H5N1 strain for the α2,6-sialic acid (SA) linkages found in the human respiratory tract (W. Peng et al. 2018); similarly, HA-T160A within the receptor-binding domain caused deglycosylation at the nearby 158 residue in several strains of H5Nx clade 2.3.4.4, creating a more hydrophobic local environment with enhanced binding to both α2,6- and α2,3-SA linkages (R. Gao et al. 2018). Similar results were found by Gu *et al.*, who found that the mutation enhanced transmission between guinea pigs (M. Gu et al. 2017).

An *in vivo* study by Peng *et al.* involved passaging HPAI H5N6 avian influenza virus in mice ten times, analysing virus after each passage for mammalian-adaptive mutations. Observed mutations included HA-A150V, NA-R143K and -F147E (both in the 150-loop adjacent to the catalytic site of

neuraminidase), and an A343T mutant in the PB2-binding site of the PA polymerase subunit. This last mutation arose after only two passages in mice and has been shown to increase growth capacity in human cells (X. Peng et al. 2016). Finally, Guo *et al.* conducted a large-scale study of all available H5Nx AIV genomes, finding 29 host-shift branches with significant signals of positive selection for mammalian adaptation (F. Guo, Li, et al. 2019).

Recent mutational studies have also focused on the capacity for antigenic shift to generate vaccine-escape mutants. Li *et al.* studied the particularly fast-spreading HPAI H5Nx clade 2.3.4.4, showing that vaccines developed against the parent 2.3.4 clade do not provide full protection against these newer viruses. Several mutations were observed within the antigenic regions of the HA protein, including residues 88, 156, 205, 208, 239, and 289. Of these, the most critical were located in antigenic region B, in the membrane-distal globular head. The authors found that anti-clade 2.3.4 vaccines protect poultry from death due to clade 2.3.4.4 infection, but not from viral shedding, potentially increasing the risk of unnoticed transmission (J. Li et al. 2020).

HPAI H5Nx viruses have continued to play a substantial role in the epidemiological landscape of avian influenza over the past five years, while studies of other major players have revealed additional markers of increased pathogenicity and/or mammalian adaptation. H9N2, an LPAI that has spread worldwide and is endemic in many regions of China, Southeast Asia, and the Middle East (Shuo Liu et al. 2020), has been the subject of much recent study. Zhang *et al.* observed a PB2 mutation, D253N, that increased replicative ability of avian H9N2 in human cells, possibly by assisting with adaptation of H9N2 to growth at 37°C (Jinfeng Zhang et al. 2018). A wider-scale study of 68 total environmental H9N2 samples from Chinese poultry farms and LPMs found that an HA-I155T mutation, previously shown to be involved in human-like α2,6-SA receptor binding, was conserved in all tested strains (S. Zou et al. 2019). Sealy *et al.* examined AIV isolates from Pakistan during 2014-2016; they found a worrying increase in the prevalence of H9N2 genotype PK3, which includes an HA-A180T substitution that facilitates binding to human-like receptors (Sealy et al. 2019). Similarly, representative isolates from Chinese LPMs universally possessed an α2,6-SA binding-associated HA-226L mutation, although this residue alone was not sufficient to allow efficient replication in mice (Teng et al. 2016). The function of these mutations can often be determined from their locations in the folded protein (e.g. NA-K142E likely impacts ligand binding or catalysis), but care must be taken in the case of multifunctional mutations with more complicated interaction networks. Song *et al.* identified one such mutation in an H9N2 strain isolated from diseased chickens in China: an observed HA-D200N switch was found to alter the viral antigenicity, increase intra-endosomal cleavage efficiency, and increase

replication in chicken cells and embryonated hens' eggs (J. Song et al. 2020). Li and colleagues looked more broadly at the reassortant potential of H9N2 when coinfecting with another common avian strain, H4N6. They coinfected 26 chickens and subsequently isolated nine reassortants from six genotypes over the next week. Some of these reassortants displayed enhanced virulence, with the H4N6 PA gene contributing to higher polymerase activity and faster viral replication (Xuyong Li et al. 2018).

Targeted virological studies of AIV sublineages can also provide valuable information on the phenotype and infective characteristics of growing populations within a given class of AIV. Peacock *et al.* used biophysical assays to check for potentially zoonotic mutations in the BJ94 and G1 lineages of H9N2, finding that the latter displayed preference for human-like receptors. This was primarily mediated by HA-A190E/D and -I227Q mutations (Peacock et al. 2017). In a similar study, Pu and colleagues studied the H9N2 G57 genotype, where a reassortment event introduced the M gene from a quail-origin virus, leading to enhanced viral transcription and protein production. The primary residues involved in these functions were M1-37A, -95K, -224N, and -242N, along with M2-21G (Pu et al. 2017). Genotype G57 remains a concern, as reassortments between this virus and H7N9 are known to have contributed to the severe zoonotic outbreaks caused by the latter virus in China during 2016-2017 (Shuo Liu et al. 2020).

H7N9 is another widespread LPAI AIV with demonstrated potential for disease burden in both poultry and humans. During a 2013-2017 series of outbreaks in China, an HPAI strain unexpectedly emerged in Guangdong, causing several human infections (Artois et al. 2018). The genome and subsequent properties of this virus have attracted significant attention, as this event illustrated the speed with which unanticipated AIV reassortants can acquire novel properties *in vivo*. Many relevant mutations originated in H9N2, as discussed above, but researchers have identified several novel mutations unique to H7N9, including PA-V100A and PB2-K526R, -A588V, -D627K, and -D701N (Pu et al. 2017; Qi et al. 2017). PB2-D701N, in particular, is a multifunctional mutation known to increase viral genome transcription and enhance viral binding to importin α, both leading to increased replication in mammalian cells (L. Liang et al. 2019; Z. Yu et al. 2019). de Vries *et al.* conducted molecular analyses to determine the minimal set of mutations necessary for avian H7N9 to acquire human receptor-binding specificity. They found two separate triple-mutants – HA-V186G/K+K193T+G228S and HA-V186N+N224K+G228S – could accomplish this switch. Both bound preferentially to extended branched N-linked glycans that end with an α2,6-Gal, a property shared with the H1N1pdm09 strain responsible for the 2009 pandemic (de Vries et al. 2017). The HA of avian H3N2 virus appears to have

evolved similar binding preferences, which potentially increase avidity by simultaneously binding two subunits of one HA trimer (W. Peng et al. 2017).

Wu *et al.* assessed adaptation in avian H7N7 via serial lung-to-lung passages in mice, finding a range of mutations over ten passages. Of particular interest were HA-D103N, close to the 130-loop, NA-K142E, near the active site of the protein, and PB2-D701N, which has previously been associated with increased virulence in H1N1, H5N1, H7N9, and H10N7 across a range of species (H. Wu et al. 2020). Herfst *et al.*, meanwhile, examined an avian H10N7 strain that caused a 2014 outbreak in North European seals, signalling a possible propensity for mammalian transmission. They observed polymorphisms in the 220-loop of the virus's HA protein, the most important of which was a Q226L mutation previously observed in the 1957 (H2) and 1968 (H3) pandemic strains. Transmission characteristics were unclear, since seals are highly social animals, but the H10N7 strain was found to transmit via respiratory droplets between ferrets, likely owing to HA-T244I and -E74D dual mutations (Herfst et al. 2020; Bodewes et al. 2015). H6N1 AIV from Chinese LPMs has also recently shown characteristics of human receptor binding, including residues HA-186L, -190V, -222A, and -228S (Tzarum et al. 2015). Hsieh *et al.* conducted an in-depth study of the binding properties of this subtype, finding the positively charged HA-R201 residue is required for binding to host cells (Hsieh et al. 2019).

Antiviral resistance is another clinically important trait associated with specific mutations within the avian influenza genome. Song *et al.* found that the M2-S31N mutation, associated with amantadine resistance, was present in three different avian H9N2 isolates from Shandong Province layer chicken farms (Y. Song et al. 2019). This particular mutation was also observed in both avian and human isolates of the HPAI H7N9 virus that caused significant outbreaks in China during 2013-2017 (Qi et al. 2017). Schaduangrat *et al.*, studying the occurrence of avian H5N1 "quasispecies" with resistance to oseltamivir, found that such resistance mutations (mainly NA-S236F) may be able to occur naturally without prior exposure to the drug (Schaduangrat et al. 2016).

Swine Influenza Virus

Due to the interactions between domestic swine and poultry and the permissive nature of swine to infection by avian influenza viruses, many of the virological studies described above are applicable to influenza strains that may spread from birds to pigs (e.g. H1N1, H3N2, H4N6, and many others). SIV antigenic drift within infected pigs has historically been thought to occur only at a low rate due to the short lifespans of these animals and the typically acute nature of SIV infections. Multiple recent studies

have, however, shown remarkable genetic diversity even within individual SIV sublineages; Ryt-Hansen *et al.*, for instance, studying H1N2 SIV, found a relatively high mutation rate of $7.6x10^{-3}$ substitutions/site/year in the HA gene, with most occurring in the globular head and antigenic sites (Ryt-Hansen et al. 2020).

The aforementioned permissiveness of pigs to infection by avian influenza viruses allows swine to act as a "mixing vessel", facilitating reassortment events during co-infections with avian, swine, and/or human influenza viruses. Zhang *et al.* conducted a study examining viral mutations that allow transmission from birds to pigs (and subsequent transmission between swine), focusing on H4N6 AIV that has twice been detected in North American swine. Four out of 115 tested avian isolates were capable of replicating effectively in swine cells, and these shared the mutations HA-Q226L and -G228S (X. Zhang et al. 2020). Such studies expand our understanding of cross-species transmission and may allow us to detect potentially zoonotic strains by identifying the presence of associated mutations in circulating SIV strains. Lai *et al.* studied the interplay between the activities of the HA and NA proteins in H1N1pdm09, finding that HA binding enhanced the cleavage of α2,6-SA by NA and potentially contributing to human adaptation (Lai et al. 2019). These findings were in line with earlier findings from Du and colleagues, who examined NA enzymatic activity in H5N1 AIV and H1N1pdm09 (Du et al. 2018). They found NA activity to be altered as expected by mutation of contact residues (in the 370, 400, and 430 loops) but also by noncontact residues in these same loops.

Anderson *et al.* conducted a sweeping phylogenetic analysis of thousands of viral gene segments from H1 and H3 SIV subtypes sourced from the USDA Influenza A Virus Surveillance System, looking for novel subtypes and evidence of antigenic drift. They detected a minor clade, H1γ-2, consisting of 37 H1 viruses isolated between 2003 and 2013. Though representing only ~1% of swine H1 genes sequenced over the past ten years, it was found to be antigenically distinct from major H1 clades, raising concerns about the proliferation of small, understudied SIV populations and their capacity to escape contemporary vaccines (Anderson et al. 2015). Lewis and colleagues conducted a similarly large-scale study of antigenic diversity in swine H1 and H3, here using data from the European ESNIP3 program, collaborations with labs in Southeast Asia, and expanding datasets from North America to encompass over 500 virus isolates. They found that SIV diversity was primarily driven by reverse zoonosis and subsequent antigenic evolution, while the contribution of avian introduction was unexpectedly limited to only one case of cross-species transmission (N. S. Lewis et al. 2016). Their results stress the need to match SIV vaccines with circulating strains, combining data from virological and epidemiological studies to track particularly concerning strains and anticipate their spread.

Equine Influenza Virus

Equine influenza viruses (EIV) spread primarily through international trade and recreational events that bring animals from different regions into close proximity. Common subtypes have included H7N7 and H3N8, although the former is now very rarely isolated and considered possibly extinct. Kumar *et al.* provide data attributing this shift to several properties of H7N7 viruses, including high codon usage bias, low mutation pressure, and less adaptation to the tRNA pool of equine cells compared to H3N8 (N. Kumar et al. 2016). H3N8 EIV is now widespread across the world, and regular outbreaks continue to occur, with varying degrees of veterinary impact on infected animals. Lee *et al.*, for instance, found that the KG11 strain isolated from Korean horses in 2011 contains a naturally truncated NS1A coding region within its NS gene, leading to impaired inhibition of host interferon production and attenuated replication (Jihye Lee, Park, and Min 2017). EIV has historically not been responsible for human infections, but horses remain a potential source of antigenic diversity generation, and indeed cross-species transmission has been detected – specifically transmission from horses to dogs. He *et al.* examined this event in detail, describing the emergence in 2002 of an H3N8 canine influenza virus (CIV) via a reassortant virus of Florida-1 clade H3N8 EIV. This CIV then spread efficiently and remained enzootic, evolving and diverging into 5-6 sublineages via intra- and inter-lineage reassortment (He, Li, Wang, et al. 2019).

Influenza Virus in Companion Animals

Such cross-transmission events have attracted recent attention to CIV, which has historically remained understudied due to the rarity of canine-to-human zoonosis. Dogs are susceptible to a wide range of influenza viruses, however, and given the high risk of contact with other species including birds and humans, virological research into CIV may provide insights into potentially hazardous reassortants with cross-species or zoonotic potential (He, Li, Wang, et al. 2019; Kalhoro et al. 2019). H3N2 CIV, for instance, first emerged in China during 2005-2006, but numerous reassortants have already been detected, including one carrying gene segments from human H1N1pdm09 (Parrish, Murcia, and Holmes 2015). Canine H3N2 was detected in the United States in 2015, prior to which only H3N8 was known to circulate widely in American dogs. H3N2 CIV possesses HA-226Q and -228G residues associated with α2,3-SA binding, in line with the mainly avian-type receptors expressed in canine respiratory tracts (Pulit-Penaloza et al. 2017), but also show a few changes linked to mammalian adaptation (e.g. HA-S159N and -W222L).

Chen *et al.*, meanwhile, conducted a study on CIV in southern China, finding two independent instances of reassortant H1N1 transmission from swine to canines in Guangxi. Both viruses contained genomic segments from North American triple reassortant H3N2, Eurasian avian-like H1N1, and H1N1pdm09 (Y. Chen et al. 2018). These H1N1 viruses have transmitted onward in dogs and reassorted with canine H3N2 to produce further variants. All tested strains contained an M2-S31N mutation that confers resistance to adamantane. Na *et al.* later studied the morphology of H3N2 CIV from China and South Korea, also finding a strain (KR07M) that carried the M gene from H1N1 and showed enhanced cellular uptake *in vitro* (Na et al. 2020).

Cats too are permissive to various influenza strains and often come into close contact with both wild birds and humans. Wang *et al.* examined an H5N6 avian virus that was isolated from cats in Guangdong in 2015, finding that its NS1 protein inhibited NF-κB and IRF2 proinflammatory activity in infected cells; this activity was attributed to its dsRNA binding domain, potentially at the S42 residue as previously observed in H5N1 AIV (L. Wang et al. 2017).

Novel Virological Assays and Technologies

Developments in experimental capabilities continue to push the boundaries of virological studies and increase the speed, efficiency, and thoroughness of influenza studies. NGS-based whole genome sequencing has expanded dramatically over the past five years, increasing the efficiency of data collection for influenza virus identification, diagnostics, and surveillance (Alleweldt et al. 2021)(Adlhoch et al. 2020; Wong et al. 2018). Concurrently, advances in computational analysis and modelling technologies are expanding the information we can gain from influenza virus genome sequences. Wu *et al.*, for instance, used sequence analysis software including the DSSP program to examine influenza virus PA protein stability under various mutational conditions, finding that the PA-K281I mutation abolishes polymerase activity due to lack of hydrogen bonding (N. C. Wu et al. 2015). Kargarfard *et al.* conducted a large-scale study applying machine learning to 674 total avian, swine, and human influenza strains, looking for reassortant changes associated with host adaptation. Algorithms including CBA, Ripper, and decision tree were used to identify statistically significant mutations, and combinations thereof, relating to host range – HA-14V, for instance, was found to be an important discriminative and combinatorial position for avian infection (Kargarfard et al. 2016). Similar machine learning techniques have also been used to score amino acid mutations predictive of avian-to-human transmission (Qiang et al. 2018). Khaliq and colleagues, meanwhile, applied

computational rule-based modelling to describe combinatorial sets of interacting residues in 12 proteins from H1N1 and H3N2 influenza viruses, also looking for host-specific signatures. They found that in H1N1, for instance, M2-G14 is associated with avian hosts while M2-E14 is associated with human infectivity (Khaliq et al. 2016). Host-specific signatures were identified in several proteins, including NP and NS1 in H1N1 and PA and PB1 in H3N2. Finally, Guo *et al.* used the CodonW software package to analyse codon bias in HPAI H5Nx subtypes, using subsequent correspondence analysis and neutrality plotting to estimate the effects of natural selection and mutation pressure on codon usage patterns (F. Guo, Shen, et al. 2019). As computational power continues to increase, it will only grow more critical to influenza virology, and the development of new analytical models and comparative testing against *in vivo* results is a critical emerging area of research.

Other technological advances involve the validation of new systems for influenza replication and protein interactions. Kasloff & Weingartl evaluated swine alveolar macrophages (IPAM 3D4/31 cells) as a host cell model for influenza virus replication due to their equal expression of α2,3- and α2,6-linked SA residues. They found that eleven tested avian-, human-, and swine-origin influenza viruses replicated optimally at 37°C, while PB2 protein amino acid profiles correlated with different growth characteristics at 33°C and 41°C (Kasloff and Weingartl 2016). Seibert *et al.* recently developed a *spol1* (swine RNA pol I promoter)-based reverse genetics system capable of rescuing both influenza A and influenza B viruses in swine and human cells. This system could limit substrate-adapted changes that may occur when candidate vaccine viruses are grown in non-natural conditions (e.g. growing swine viruses in embryonated hens' eggs) (Seibert et al. 2021). As this study illustrates, research into influenza B has increased in the past five years (along with influenzas C and D). Although these viruses have not yet caused zoonotic outbreaks, they have demonstrated their capacities for widespread and often cross-species transmission, warranting increased attention (Yan et al. 2019). Ishida *et al.* established a reverse genetics system for influenza D virus utilizing human rectal tumor cells (HRT-18G). Despite lower transfection efficiency that the HEK-293T cells commonly used for influenzas A, B, and C, HRT-18Gs produced virus with much higher infectivity (Ishida et al. 2020). Following optimization of transfection ratios with 7 viral RNA-synthetic plasmids and 4 protein expression plasmids, the resulting virus showed similar growth properties to the wild-type.

Similarly, *in vitro* methods for protein isolation and interaction studies have also been expanded over the past five years. Thulasi Raman *et al.* infected swine tracheal cells with a Strep-tagged NS1 protein, using immunoprecipitation followed by liquid chromatography with tandem mass spectrometry to characterise protein complexes and NS1 interaction partners (Thulasi Raman and Zhou 2016). Liu and

colleagues used a similar MS-based analysis to examine the phosphorylation of canine lung cell proteins after H3N2 CIV infection, finding dramatic changes in phosphoproteins associated with RNA processing and modification, chromatin remodelling, and energy production/conservation (Y. Liu et al. 2020). Nogales *et al.*, meanwhile, examined the functions of the NS1 protein of influenzas A, B, C, and D in canine MDCK and human A549 cells. They show that the B, C, and D proteins exhibited different RNA-binding properties, and IAV expressing heterotypic NS1 proteins could be used as the basis for live attenuated influenza vaccines as their replication *in vivo* is stunted (Nogales et al. 2019). Liu and colleagues used a similar MS-based analysis to examine the phosphorylation of canine lung cell proteins after H3N2 CIV infection, finding dramatic changes in phosphoproteins associated with RNA processing and modification, chromatin remodelling, and energy production/conservation (Y. Liu et al. 2020). Nogales *et al.*, meanwhile, examined the functions of the NS1 protein of influenzas A, B, C, and D in canine MDCK and human A549 cells. They show that the B, C, and D proteins exhibited different RNA-binding properties, and IAV expressing heterotypic NS1 proteins could be used as the basis for live attenuated influenza vaccines as their replication *in vivo* is stunted (Nogales et al. 2019).

Lastly, organoids are proving an invaluable tool for biological research, including for the study of interactions between IAV and human respiratory cells (J. Zhou et al. 2018). Porcine intestinal (Yang Li et al. 2020) and testicular (Vermeulen et al. 2019; Sakib et al. 2019) organoids have been developed and used successfully. However, to date, porcine lung organoids have not been reported. Similarly, chicken enteroids have recently been developed that include immune, as well as intestinal, cells, and were successfully infected with IAV (Nash et al. 2021); again, though, the development of lung organoids is lacking so far.

Current research

At the Friedrich-Loeffler-Institut, researchers are applying 3rd-generation sequencing (including Nanopore MinION technology) to whole-genome IAV analysis. Multiple aspects of influenza virology are under study at the Exotic and Emerging Avian Viral Diseases Unit of the US National Poultry Research Center, including genetic and epidemiological research on LPAI and HPAI strains from the 2020 outbreak in North/South Carolina turkey farms and on LPAI H2N2 from American LPMs. Research is also underway here into AIV adaptation and evolution, specifically studying the molecular determinants of H7 incursions from wild birds to domestic poultry and the adaptation of Mexican-lineage LPAI H5N2 in chickens. At the Southeast Poultry Research Laboratory, study of recent outbreak viruses is ongoing, with researchers applying standardized approaches to evaluate novel isolates and

measure transmissibility for outbreak preparedness. Meanwhile, scientists at the Royal Veterinary College of the University of London carry out ecological and evolutionary studies of AIV in wild birds.

Scientists at the UK Animal and Plant Health Agency are currently conducting Defra-funded research into the mechanisms of influenza virulence and species tropism and associated surveillance strategies, threat identification methodology, and outbreak control. Mammalian influenza viruses are also under specific study here, with researchers examining host/virus interactions in swine, cattle, and humans. At ANSES, scientists are examining the genetic and antigenic evolution of SIV in France, conducting in-depth genome analyses to identify molecular determinants of virulence and cross-species transmission. Methods include *in silico*, *in vitro*, and *in vivo* assays, and data is shared within the OFFLU Swine Influenza group. At the National Veterinary Research Institute in Vom, Nigeria, research is underway into IAV molecular characterization, particularly in a zoonotic context, with studies examining genotypic changes associated with transmission and pathogenesis.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of animal influenza virology should be considered priorities for future research:

- *Sequence-to-phenotype prediction models*
- *Predictive approaches for understanding the level of risk posed to poultry by new isolates*
- *Determinants of host adaptation beyond the HA and NA proteins*
- *Mechanisms/pathways of intra-host virus evolution*
- *Mechanisms of adaptation of avian and human influenza viruses to swine*
- *Determinants of the emergence of reassortant viruses*
- *Continuing development of NGS-based whole genome sequencing*
- *Effective deployment of 3rd-gen sequencing technologies for both wet- and dry-lab protocols, allowing point-of-incidence analysis*
- *Rapid characterization of variants circulating in the field via high-throughput point-of-care technology*
- *Fast typing of avian influenza isolates without virus isolation (applicable for noninvasive samples from wild birds)*
- *Expand virological research into areas of Asia where circulating strains remain understudied*

Pathogenesis

Influenza A viruses (IAV) infect a wide range of animals critical for food production (e.g. poultry and swine) or involved in recreation (horses) and human companionship (cats and dogs). Some IAV can infect multiple species, while others are restricted to a single host (Eng, Tong, and Tan 2017) – and within one host, various subtypes can have different tissue tropisms, causing diverse infection patterns, clinical signs, and viral shedding (X. Zhang et al. 2020). Gene segment swapping between co-infecting subtypes can result in dramatic shifts in IAV pathogenicity, while selective pressure induces smaller-scale changes that can nevertheless have wide-ranging effects. Due to the small size of the IAV genome, viral proteins are multifunctional and even minor changes can be synergistic, leading to unexpected changes in host or tissue tropism. Even closely-related, cocirculating influenza viruses can vary drastically in pathogenicity, and unexpected reassortments can lead to the emergence of dangerous combinatorial properties (Mei et al. 2019; Uchida et al. 2019). Influenza pathogenesis research is primarily aimed at uncovering the molecular changes behind these differences, studying determinants of viral transmission and tropism, zoonosis and airborne transmission, and interactions with host cells.

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps in animal influenza pathogenesis in 2014/15:

- *molecular determinants of virulence and immune evasion in target species*
- *determinants of high- vs. low-pathogenicity avian influenza*
- *determinants of viral shedding from respiratory vs. intestinal tracts*
- *determinants of host range and tissue tropism, particularly with cross-species or zoonotic potential*
- *mechanisms (genetic or otherwise) behind duck resistance to HPAI strains*
- *influence of virus on host cell molecular biology*
- *effects of co-infection with different viruses or bacteria*

Avian Influenza Pathogenesis

Avian influenza viruses are considered as either low pathogenic (LPAI) or highly pathogenic (HPAI) dependent on their disease burden in poultry (Lycett, Duchatel, and Digard 2019). Both types of virus can spread rapidly through infected flocks and may be capable of transmission to other species, including swine and humans, with varying clinical signs therein that may not reflect their pathogenicity in birds (CDC, NCIRD Fact Sheet). Poultry infected with LPAI strains may show minimal or even no signs of infection, allowing the disease to spread unchecked unless active surveillance is carried out within the flock. HPAI strains, conversely, tend to cause severe systemic infections and can cause mortality of up to 90-100% in chickens and other poultry (Stoimenov et al. 2019; Dinev et al. 2020). Ducks, however, tend to be asymptomatic even with HPAI infection, allowing them (along with other waterfowl species) to serve as a natural reservoir for these viruses (Smith et al. 2015a). The migratory routes of wild waterfowl bring them into contact with a wide range of domestic poultry species (Pantin-Jackwood et al. 2016). Kikutani and colleagues studied the mechanisms of duck-to-chicken transmission in H6 AIV, finding that HA-N192D and -E190V mutations facilitate the process by enhancing binding to sulphated SAα2,3Gal glycans (Kikutani et al. 2020).

The main molecular determinant of an HPAI phenotype is the acquisition of a multi-basic cleavage site (MBCS) in the HA gene (Joseph et al. 2017; Dietze et al. 2018). This site, where HA is post-translationally cleaved by host proteases, typically contains a single basic residue in LPAI strains (e.g. PQRET**R**/G) but contains two or more basic residues in HPAI strains (e.g. PQRE**RRRKR**/G). This substitution widens the pool of host proteases capable of cleaving the site, expanding tissue tropism and allowing the systemic spread characteristic of HPAI infection in poultry (Seekings et al. 2018). LPAI H7N9, for instance, caused multiple outbreaks in China before unexpectedly producing an HPAI variant via MBCS acquisition during the fifth outbreak in 2016-2017 (Tanikawa et al. 2019). Seekings *et al.* detected a rare di-basic cleavage site motif (PEIPKKRGLF) unique to LPAI H7N7 in Galliformes, and found that three distinct MBCS-containing HPAI variants had evolved from it and were cocirculating locally (Seekings et al. 2018). Kwon and colleagues, however, found that an MBCS may not be an absolute requirement for systemic spread of an AIV. They examined an H7N6 strain isolated from several organs (including lung, spleen, and brain) of a dead Korean mallard duck in 2007, finding a trypsin-independent growth phenotype dependent on N6 NA and a specific cleavage motif in HA (H. Il Kwon et al. 2019). The authors theorize that prothrombin activation by N6 NA during replication

may enable thrombin-dependent cleavage of the GRG motif, removing the need for trypsin-like enzymes and allowing systemic spread without a classical MBCS.

Studies of LPAI H9N2, which has spread throughout China, Southeast Asia, the Middle East, and Europe in recent years (Shuo Liu et al. 2020; Fallah Mehrabadi et al. 2019), have uncovered several polymorphic sites that modulate the infectivity and pathogenic phenotype of the virus *in vivo*. Li *et al.* examined mutations within the 3' UTR of the H9N2 NA gene, finding that the NA-U13C mutation promotes viral genome expression (Xi Li et al. 2019). Within the coding sequence, NA-G127S and -S450L mutations have also been shown to increase pathogenicity – isolates with these mutations display mortality >75% despite their classification as LPAI (Shahzad et al. 2020). Ismail and colleagues found that H9N2-infected chickens displayed more severe disease (higher viral titres and increased lesions on internal organs) after vaccination with infectious bronchitis live attenuated vaccine, raising the possibility of a deleterious immune interaction (Ismail et al. 2018). Meanwhile, James *et al.* studied the PB1 viral polymerase subunit of H9N2, finding that a full-length PB1-F2 protein is associated with prolonged viral shedding and increased minimal lethal dose, both facilitating transmission. This full-length product is found in ~93% of avian isolates but is frequently truncated in mammalian isolates, suggesting mammalian adaptation (James et al. 2016). Co-infection studies have also dealt frequently with H9N2; Belkasmi *et al.* examined co-infection of chickens with H9N2 AIV and Moroccan Italy 02 infectious bronchitis virus, finding that the trypsin-like serine domain encoded by the latter enhances H9N2 pathogenicity (Belkasmi et al. 2020). Fowl adenovirus also appears to play a complementary role with AIV, with FAdV-4 causing immunosuppression that inhibits antibody responses to inactivated H9 vaccines (Niu et al. 2017). In bacterial infections, enhanced pathogenicity has been observed during coinfections of H9N2 with *E. coli* in chickens and *Pseudomonas aeruginosa* in commercial mink (which are susceptible to H9N2 AIV infection) (Bo-shun et al. 2020).

Much recent attention has been focused on HPAI H5Nx viruses that have spread rapidly worldwide over the past five years. These viruses are spread primarily by migratory waterfowl which are often resistant to disease but shed relatively high titres for at least seven days post-infection (Pantin-Jackwood et al. 2016). In chickens and other domestic poultry, HPAI H5Nx viruses can cause severe pathologies, spreading into the endothelium, central nervous system, and myocardium and inducing systemic inflammation with high mortality (Butler et al. 2016). Several hundred human cases have also been reported with ~50-60% mortality, but human-to-human transmission has not yet been observed (Mahardika et al. 2019). Azab *et al.* studied an early mutational event – the shift from H5 clade 2.2.1 to clade 2.2.1.2 that occurred in 2014/2015 – finding that point mutations in the MBCS likely played a

role in the higher virulence of the latter clade (Azab et al. 2017). Guo and colleagues, meanwhile, examined the emergence of the particularly fast-spreading clade 2.3.4.4 from the older clade 2.3.4. They found mutations HA-K222Q and -S227R, unique to HPAI H5 proteins, facilitate binding to fucosylated sialosides. These substitutions seem to have appeared at the root of H5Nx clade 2.3.4.4, as K222 and S227 are highly conserved in all clades of the earlier HPAI H5N1. This poultry-specific adaptation may have facilitated the rapid spread of clade 2.3.4.4 across Southeast Asia, Europe, and North America (H. Guo et al. 2017).

HPAI H5N8 caused two recent outbreaks in Europe – one in 2014-2015, and another 2016-2017. The latter outbreak showed substantially higher virulence and pathogenicity, with a greater number of affected poultry farms and higher mortality. Vigeveno *et al.* examined the determinants for this change, finding that extensive reassortment had occurred in the viral polymerase genes between outbreaks. The polymerase complexes from the first outbreak showed much higher activity in avian cells but also induced higher levels of IFN-β via Retinoic acid-Induced Gene I (RIG-I) activation – although activity of the polymerase from the virus causing the second outbreak was lower, a corresponding drop in innate immune activation overbalanced this change, resulting in the observed increase in virulence (Vigeveno et al. 2020). In Taiwan, after years of dominant LPAI H5N2 circulation, HPAI H5Nx clade 2.3.4.4 viruses emerged in 2015 and are now circulating widely in the local poultry industry. Wang and colleagues looked specifically at the role of the viral NS1 protein in HPAI H5Nx pathogenicity, finding that deletions in the highly variable NS1 C-terminus are associated with higher proinflammatory cytokine mRNA production. PDZ-binding motifs within the C-terminus also modulate pathogenicity via interactions with PDZ domain-containing host proteins that modulate cell polarity establishment and tight junction formation (W. C. Wang et al. 2020). The NS1 protein in general has been found to display remarkably high plasticity, contributing to immune evasion and adaptation across various hosts by following random evolutionary pathways within various influenza A lineages (Muñoz-Moreno et al. 2019).

Such host cell interactions are a critical determinant of viral virulence and play an important role in initiation of infection, immune activation, and viral replication and spread. Chothe *et al.* conducted a wide-spectrum study of immune dynamics in chicken primary lung cells and macrophages, finding that NLRC5, the largest member of the NOD-like receptor family, plays a proviral role by decreasing innate cytokine gene expression in response to LPAI and HPAI infection (Chothe et al. 2020). Such modulation of the immune system is an important factor in virulence – Kumar *et al.*, for instance, found that LPAI AIV strain infection leads to upregulation of antiviral genes (e.g. *IFIT5* and *ISG12*) and β-defensins,

while HPAI strains exhibit a more proinflammatory activity by upregulating chemokines, like *CCL4* and *CXCR4*, and IFN-stimulated genes (e.g. *STAT3* and *TGFB1/3*) (A. Kumar et al. 2017). Drobik-Czwarno and colleagues took an opposite approach, looking for genetic determinants of HPAI AIV resistance/survival in commercial egg layer chickens. Several regions in chromosomes 1, 7, 9, and 15 coding for membrane receptors and/or involved in immune responses were associated with H5N2 survival, while associated regions for H7N3 were neuronal cell surface, signal transduction, and immune response genes in chromosomes 1 and 5 (W. Drobik-Czwarno et al. 2018). In ducks, meanwhile, PIAS2 (Protein Inhibitor of Activated STAT2) expression was linked to HPAI H5N1 strain DK212 replication through its SUMO E3 ligase activity (Zu et al. 2020).

Swine Influenza Pathogenesis

Swine are highly susceptible to a wide range of influenza viruses and can host dangerous reassortments between coinfecting strains (Vincent et al. 2017). Chastagner *et al.*, for instance, describe the emergence of a triple-reassortant H1N2 SIV with an avian-like backbone, N2 from a human seasonal H3N2 isolate, and M segment from H1N1pdm09 (Chastagner, Bonin, et al. 2019). Such reassortments can produce viruses with the potential for zoonotic or cross-species transmission. Yang *et al.*, for instance, passaged H9N2 AIV three times in differentiated swine airway epithelial cells and found a PB2-G685R mutation that, in combination with HA-A190V or -T212I, significantly increased virulence in mouse models (W. Yang et al. 2017). Veljkovic and colleagues conducted an *in silico* analysis of A/swine/Guangdong/104/2013 (H1N1), finding strong human infectivity and potential to acquire a human-adaptive HA1-D74N mutation (Veljkovic et al. 2016).

The development of an *in vitro* system for detailed study of the interaction between SIV and the porcine respiratory epithelium represents an important advance. Wu *et al.* have designed and tested an air-liquid-interface long-term culture method using primary epithelial cells from porcine bronchi; following IAV infection, the epithelium exhibited apoptosis which was associated with loss of cilia and epithelial thinning (N. H. Wu et al. 2016). Thomas *et al.* also reported the suitability of the respiratory epithelial cell line, MK1-OSU, for *in vitro* study of SIV pathogenesis (Thomas et al. 2018).

In the densely-populated environment of pig farms, multiple circulating pathogens are common, and coinfections can lead to unexpected pathological and immunological interactions. Simultaneous infection with H1N1 SIV and *Haemophilus parasuis*, a common bacterial pathogen of swine, increases

replication of both pathogens and induces stronger local and systemic immune responses. Nasal shedding of SIV, and lung lesions due to *H. parasuis*, were also increased (Pomorska-Mól et al. 2017).

Mammalian Adaptation

Mammalian adaptations – mutations that allow avian viruses to replicate and thrive in swine or human hosts – are required for cross-species transmission and zoonosis. Identification of such mutations allows us to monitor their emergence in circulating strains, increasing our preparedness for potential outbreaks and informing vaccine development. Yamaji *et al.*, for instance, found five substitutions in the PA polymerase subunit of a Vietnamese HPAI H5N1 human isolate – PA-V44I, -V127A, -V241Y, -A343T, and -I573V – that increased growth capacity in A549 cells and pathogenicity in mice (Yamaji et al. 2015). A similar study focused on the H5 HA antigenic protein, where human-adaptive mutations (e.g. HA-N224K and -Q226L) have been found to decrease heat stability, reduce airborne transmission potential, and raise pH of fusion >5.5 (whereas a fusion pH of $\leq$ 5.5 confers optimal infectivity in human cells). HA-T318I and -H110Y served as compensatory mutations, restabilizing the HA protein while retaining human-type receptor binding preference (Hanson et al. 2016). Comparable results were found in H1N1pdm09 SIV, where experimentally-introduced destabilizing mutations reverted during mammalian adaptation and replaced with stabilizing, fusion pH-lowering substitutions like HA2-R106K (Russier et al. 2016). Swine in general appear to support a wide range of HA stabilities/pH's of fusion, in keeping with their reputation as a melting pot of influenza strains from various species, and many recent H1N1 and H3N2 SIV isolates include stabilized (human-like) HA proteins (Russier et al. 2017).

HA mutations can also alter growth characteristics in mammalian cells – a study of duck AIV adaptation to swine respiratory cells, for example, identified HA2-G67S and -S113F as conferring a fast-growth phenotype by enhancing HA-mediated membrane fusion (Bourret et al. 2017). Deletion of HA glycosylation sites, previously observed in circulating HPAI H5N1 strains (X. Zhang et al. 2015), may also play a role in mammalian virulence, with mutations at H5 HA-144 and either -158 or -169 causing enhanced proinflammatory cytokine release and ER stress in human cells and mouse models (Y. Yin et al. 2020). Jegede and colleagues passaged LPAI H9N2 AIV 19 times in chickens via aerosol inoculation, finding selection for the HA-Q226L mutation associated with human receptor-binding preference (HA-226Q to -226L ratio increased from 54:46 in the first inoculum to 0:100 at passage 19). This suggests that ostensibly mammalian-adaptive traits may be acquired during asymptomatic circulation in poultry (Jegede et al. 2018).

The surface-exposed HA is a major player in influenza virulence and pathogenicity, but internal genes also play a significant role in mammalian adaptation. Bogdanow *et al.* found that splicing of the IAV genome M segment is a host range determinant, with eight nucleotides in the 3' splice site responsible for modulating replication efficiency in bird- vs. human-adapted strains (Bogdanow et al. 2019). In the PB2 polymerase subunit gene, many mutations that influence mammalian pathogenicity have been identified and are observed at high frequencies in human influenza viruses (C. Y. Lee et al. 2020). Lee and colleagues found the most potent of these to be PB2-K526R, although its function remains unclear. Also highly potent were E627K, which interacts with the host factor ANP32A to stimulate RNA synthesis, and D701N, which may reflect the importance of PB2 nuclear localization via interaction with importin-α (C. Y. Lee et al. 2020). Chin *et al.* also found a high degree of plasticity in PB2-701 along with -702, with the combinations -701A/702E and -701N/702G broadening tissue tropism *in vitro* and *in vivo* (Chin et al. 2017). Gain-of-function potential has also been observed in the structural NP gene, where a K470R mutation in HPAI H5N1 increased mammalian virulence by increasing polymerase activity and replication. This residue is surface-exposed in trimeric NP and was found to be polymorphic among IAVs (Lin Chen et al. 2017).

Recent HPAI H5N8 viruses of the now-dominant clade 2.3.4.4 have demonstrated lower pathogenicity in mammalian hosts compared to older HPAI H5N1 viruses (clade 2.2). Park and colleagues generated single-gene substitutes to probe this change, finding that HPAI H5N1 PB2 and NA genes significantly increased pathogenicity; the former, in particular, increased virulence in mice 1000-fold and substantially raised polymerase activity (S. J. Park et al. 2018).

Influenza D Virus

Recent studies have begun to bring focus to influenza D virus (IDV), an influenza genus first identified in 2011. Although originally found in pigs, IDV is now known to circulate primarily in cattle, where seroprevalence is high (Bailey, Choi, et al. 2018). Alongside, the incidence of swine IDV infections may be increasing (Bailey, Fieldhouse, et al. 2018), highlighting the need to keep a close watch on this potentially dangerous genus. An early genetic analysis of IDV by Collin *et al.* looked at six viruses isolated from cattle with bovine respiratory disease, finding that the HA-212K/R residue appeared to be an antigenic determinant (Collin et al. 2015). Experimentally-infected cattle have been shown capable of transmitting IDV to naïve counterparts via in-pen contact (Ferguson et al. 2016). Knowledge of cross-species infectivity is limited but contact transmission of IDV is possible between guinea pigs. The infected animals show no clinical signs, however, despite seroconversion and presence of viral

antigen in the lungs (Sreenivasan et al. 2015). Ferguson *et al.*, meanwhile, found that ferrets can be experimentally infected with IDV but are not infected by contact with contaminated fomites (Ferguson et al. 2016).

Ongoing research

Erasmus MC Viroscience maintains several lines of pathogenesis research, including studies of the genetic and phenotypic determinants of mammalian virulence and the emergence of HPAI from LPAI AIV strains. At the US National Poultry Research Center, meanwhile, researchers are studying the pathobiology of AIV isolates selected based on a risk assessment of their threat to poultry (e.g. from recent US outbreaks), conducting tests of infectivity, viral transmission and shedding, morbidity/mortality, and determinants of tissue tropism. In this centre's Exotic and Emerging Avian Viral Diseases Unit, scientists are examining the genetic determinants of mallard virulence in HPAI H5N8 clade 2.3.4.4, where the two strains responsible for the 2014-2015 and 2016-2017 outbreaks caused markedly different clinical signs (with the latter exhibiting severe pathogenicity and high mortality). Future studies at this unit will focus on molecular markers of infectivity, pathogenicity, and transmissibility of AIV and host-specific factors associated with pathogenic profiles in birds of various physiological states.

Researchers at ANSES in France study the role of SIV within the larger porcine respiratory disease complex, where influenza virus may coinfect with other viral and bacterial pathogens and lead to significant health complications. At Ghent University, researchers are currently studying determinants of cross-species transmissibility, using pig infection models to determine (a) the genetic changes required for adaptation of H3N2 AIV to swine, and (b) the involvement of host factors in viral pathogenesis and transmission to pigs or ferrets.

At the University of Nottingham, UK, researchers continue to study molecular determinants of equine influenza (including HA polymorphisms and the role of NS1 in virulence and cross-species transmission), while in Germany at the Friedrich-Loeffler-Institut, scientists are conducting pathogenesis studies of IAV in numerous animal models, including birds and mammalian models like swine, ferrets, and mice.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of animal influenza pathogenesis should be considered priorities for future research:

- *Molecular determinants of human adaptation/zoonosis and subsequent human-to-human transmission*
- *Increased understanding of the role of pigs in the emergence of human pandemic strains*
- *Viral, host, and environmental factors that influence the risk of acquiring an HA multibasic cleavage site*
- *Duration of infectious windows for various subtypes*
- *Determinants of virulence, particularly in waterfowl, apart from HA cleavage site*
- *Mechanisms behind increased pathology observed with some H9N2 and H5N2 LPAI strains*
- *Further study of new HPAI H5N8 and H8Nx strains and their potential for reassortment and zoonosis*
- *Improved methods for zoonotic risk assessment (e.g. cell culture, animal models)*
- *Emergence of HPAI from LPAI subtypes*
- *Potential role of the vasculature in HPAI pathogenesis*
- *Comparative studies of pathogenesis with different influenza viruses in the swine host*
- *Standardization of pathogenesis/transmission models and experimental influenza delivery routes across laboratories*

Immunology

The immune response to influenza virus infection operates at the innate and adaptive level both to tackle acute infection, and, in the case of adaptive immunity, to generate long-lasting immune memory. Pattern-recognition receptors act as sentinels, detecting the earliest stages of viral incursion and activating downstream signalling cascades that amplify the alarm call. Type I interferons (IFN) are key immune molecules that protect cells from infection and also support the activation of innate and adaptive immune cells to combat the infection. Soon after, B cells are stimulated to produce antibodies that recognise several influenza virus proteins and so bind to the viral surface, interfering with infection of cells; alongside, T cells recognising influenza peptide epitopes bound on major histocompatibility complex (MHC) I or II can destroy infected cells, preventing the virus from completing its life cycle, or may focus instead on cytokine production and supporting the anti-viral action of B cells. In parallel, influenza virus has developed multiple mechanisms of immune evasion that affect the ensuing host response (see Pathogenesis).

Understanding the multiple aspects of the immune response to influenza virus infection is a significant challenge. Moreover, many of the components of the immune response are influenced by the genetic background of the host in ways that we are only just beginning to understand. Different breeds within the same species, as well as different species, also respond differently to infection with the same virus; ducks for example exhibit strong clinical resistance HPAI, while chickens do not. Understanding the mechanisms underpinning susceptibility versus resistance is an active area of influenza research.

The design of effective cross-protective vaccines is also reliant on a thorough understanding of natural infection and immunity. In particular, activation of local immune responses in the lung mucosa and stimulation of cross-reactive T cells seem to be key features of broadly-reactive immunity that must be recapitulated by the next generation of vaccine candidates. Understanding the drivers of long-lasting immune memory in different species will also be important.

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps in animal influenza immunology in 2014/15:

- *contribution of host immunogenetics in innate protection, including by the generation of transgenic animals to study virus-host interactions*
- *immune correlates of protection, and tests for them*
- *identification of B and T cell epitopes that provide best protection from inactivated and vectored vaccines*
- *design of systems to predict escape mutants to allow rapid development of vaccine seed strains*
- *existence of carrier state in some species (pheasants) or immunocompromised animals*
- *mechanism of action of duck RIG-I in conferring AI resistance, and whether there are homologues in other avian species*
- *timing and identity of cytokines expressed following AI infection that reduce virus shedding from cells and increases resistance of birds to infection*
- *role of cell mediated immunity in protection following infection in wild birds versus poultry, and in particular its role in determining transmission*
- *Identification of antibody epitopes important for antigenic drift in swine and development of models to predict epitopes based on HA sequence evolution*
- *Identification of conserved B- and T- cell epitopes within and between virus subtypes for targeting by vaccines*
- *Identification of subtype-specific B- and T- cell epitopes for subtype diagnosis of previous infection*

Literature review

Avian Influenza Immunology

Host Immunogenetics

Although commercially reared chickens are renowned for their high susceptibility to HPAI, there are marked differences in the level of susceptibility between breeds. An *et al.* compared the genetic characteristics of the more-susceptible Leghorn GB2 and less-susceptible Fayoumi M43 breeds and uncovered a high degree of genetic difference in the anti-viral *MX-1* and heat-shock protein genes (An et al. 2019). Moreover, the regulation of transcription and methylation of these genes was significantly more stable in Fayoumi birds than in the Leghorns during AIV infection (An et al. 2019), raising the possibility that genomic stability is a differential feature linked to susceptibility in chicken breeds. It

should be noted, however, that this study was conducted with small numbers of highly inbred laboratory chickens, and so the results will require validation in larger cohorts under field conditions. In fact, a retrospective genome-wide association study seeking to identify genetic features associated with the survival of chickens from natural infection with HPAI H5N2 during the 2015 US outbreak concluded that resistance was indeed a complex trait mediated by multiple genes (Wioleta Drobik-Czwarno et al. 2018). Similarly, Susanti *et al.* uncovered marked Mx genotype variation between individuals within breeds (Susanti et al. 2017). Therefore, the idea that there exist resistant versus susceptible chicken breeds is perhaps not as reliable, at least at the individual animal level, as was first thought.

There is also some evidence of a link between breed and the magnitude of CD8 T-cell responses, which is associated with the susceptibility of chickens to AI infection. Blohm et al. found that two phylogenetically related brown layer breeds had increased survival following infection with LPAI or HPAI and relatively higher CD8 T-cell counts during infection than the more susceptible white layer breeds (Blohm et al. 2016). Importantly, the more resistant breeds exhibited a generally more active CD8 T-cell compartment, as these hens also eliminated injected allogeneic cells more rapidly than their less AI-resistant counterparts (Blohm et al. 2016). At present, the genetic basis for this differential CD8 T cell reactivity is unknown and could be important to define to support the development of disease-resistant commercial chicken breeds.

The immunogenetic basis of the resistance of ducks to AI infection has also begun to be investigated. Recent research has revealed high MHCI diversity in wild duck populations, which is not present in domesticated ducks and would be expected to offer greater population-level resistance to AI, whilst also placing the virus under continual pressure to evolve (Fleming-Canepa et al. 2016). It is interesting to speculate that the higher level of inbreeding in commercial poultry breeds would remove some pressure from the virus, and may contribute to their relatively increased level of AI susceptibility than their counterpart wild species.

Interferon-induced transmembrane protein 3 (IFITM3) has broad anti-viral properties and restricts the replication of influenza virus in cells *in vitro* (Brass et al. 2009). Polymorphisms in this gene in humans have been associated with differential susceptibility to influenza H1N1 infection (Xuan et al. 2015), leading researchers to ask whether the same was true in avian species. Kim et al. discovered 14 genetic polymorphisms among commercially reared Delkab White and Ross breeds of chicken, of which two were predicted to be deleterious to the function of IFITM3 (Y. C. Kim, Jeong, and Jeong 2019). The

authors also noted structural differences within the C-terminal domain of chicken and duck *IFITM3* (Y. C. Kim, Jeong, and Jeong 2019), which may warrant further investigation.

Innate Immunity to Avian Influenza

Understanding the clinical resistance of some avian species to influenza subtypes that are rapidly fatal in others, including commercial poultry, is a key area of research. The RIG-I protein and its binding partners have previously been identified as dominant factors underpinning the resistance of ducks to HPAI (Barber et al. 2010a; Smith et al. 2015b). Moreover, a recent study also identified a role for TRIM32. In mammals, the E3 ubiquitin ligase tripartite motif 32 (TRIM32) is an important regulator of the innate antiviral response; during IAV infection of human cells and cell lines *in vitro,* TRIM32 restricted viral replication by targeting the influenza polymerase basic protein 1 (PB1) for degradation (Fu et al. 2015). Wu et al. have now identified and studied this molecule in ducks (S. Wu et al. 2020). TRIM32 mRNA expression was increased in the lungs and spleens of H5N6 HPAI-infected ducks at 3 days post-infection, and *in vitro*, duck TRIM32 increased IFNβ production (by interacting with the innate immune adaptor protein STING) and reduced H5N6 replication (S. Wu et al. 2020). It is possible that TRIM32 also functions to transiently increase the expression of innate immune response genes, including those encoding IFNα, IL-1β, RIG-I, Mx, and OAS, as seen in duck embryonic fibroblasts infected with duck Tembusu virus *in vitro* (T. Li et al. 2021); however, it is unclear whether the same pathways operate in the context of influenza virus infection or *in vivo*. Wu *et al.* also identified TRIM32 sequences in chicken and goose genomes (S. Wu et al. 2020), but these species are typically highly susceptible to HPAI infection. Further studies are needed to dissect the role of TRIM32 in AI-susceptible and resistant species to understand whether/how it contributes to their susceptibility.

TRIM27 may also have a role in mediating the resistance to AI infection in ducks. The *TRIM27-L* gene is present in ducks but not chickens, and its expression is increased in lung tissues during infection with LPAI or HPAI; moreover, when overexpressed in chicken DF-1 cells *in vitro* in the presence of duck RIG-I, TRIM27-L induced strong activation of *MX1* and *IFNβ* transcription (Blaine et al. 2015).

In addition to the known mediators of innate responses to viral infection, transcriptional regulatory long non-coding RNAs (lncRNAs) might have an emerging role. Duck tissues exhibited significantly altered expression of eight lncRNAs with predicted innate immune-regulatory functions during H5N1 HPAI infection, suggesting a possible role for these molecules in the antiviral response (C. Lu et al. 2019).

While ducks display marked resistance to multiple AI subtypes, including most HPAI viruses, chickens are highly susceptible to HPAI, often succumbing within a few days of infection. Aside from the lack of genes encoding RIG-I and the RIG-I binding protein RNF-135 (Magor et al. 2013; Barber et al. 2010b), few determinants of this differential susceptibility had been identified, due, in part, to a lack of side-by-side studies in chickens and ducks. To address this, Smith *et al.* infected ducks and chickens with HPAI H5N1 and found clear differences in their expression of the known anti-viral interferon-induced transmembrane protein (IFITM) gene family (Smith et al. 2015c) *IFITM 1,2,3,5 and 10* genes were identified in both species, with levels of protein homology ranging from 40-92%. However, while H5N1 infection of ducks led to an up to 93-fold increase in the *IFITM 1-3* expression level in the lung and ileum as early as day 1 post-infection, suggesting high levels of upstream interferon induction, infected chickens only showed a slight increase in *IFITM3* expression in the ileum on day 3 (Smith et al. 2015c). Further studies of the role of IFITM family members in other avian species and IAV subtypes may provide much-needed insights into differential AI susceptibility.

In ducks, RIG-I is a primary sensor of viral RNAs and triggers downstream induction of Type I IFNs. Until recently it was unclear which molecules fulfilled this role in chicken cells. Toll-like receptor (TLR) 3 and melanoma differentiation-associated protein 5 (MDA5) can both sense viral RNAs and are present in chicken cells, but data from a recent *in vitro* study suggest that TLR3 is dispensable for the induction of type I IFN in response to influenza virus infection, which relies instead on MDA5 (S. bin Lee et al. 2020). Chicken embryonic fibroblasts overexpressing MDA5 and infected with HPAI H5N6 exhibited significantly increased expression of the IFN-stimulated genes *MX1, IFI6, IFIT5, RSAD2,* and *OASL* (S. Yu et al. 2020).

The downstream adaptor of RIG-I, STING, contributes to antiviral immunity in ducks (Y. Cheng et al. 2019), and also seems to be activated in a RIG-I-independent manner in chicken cells. DF-1 cells infected with H9N2 LPAI increased expression of STING mRNA, and cells overexpressing STING exhibited induced IFNβ promotor activation and pro-inflammatory cytokine induction (Y. Cheng et al. 2015). Moreover, STING overexpression reduced AI replication in DF-1 cells, and was shown to interact with MDA5, which the researchers proposed represented a functional compensation for the lack of RIG-I in these cells (Y. Cheng et al. 2015). The possible role of RIG-I-independent STING activation *in vivo* in chickens is unknown and warrants further investigation.

In *in vitro* studies, IRF7 knockout chicken embryonic fibroblasts supported higher levels of LPAI replication (T. H. Kim, Kern, and Zhou 2020). Also *in vitro*, chicken cathelicidin-B1 – a member of the cathelicidin family of host defence proteins – was shown to agglutinate several IAV strains, inhibiting their ability to infect cells (L. Peng et al. 2020). Similarly, the innate immune mediator mannose binding

lectin from chickens inhibited IAV haemagglutination *in vitro* (Weidong Zhang et al. 2017). Evaluating the relevance of these pathways and molecules *in vivo* may advance our understanding of anti-viral immunity and therefore open up new avenues of investigation into novel anti-influenza therapeutics/immunomodulators.

Although the vast majority of studies have focused on host immunity to influenza, several have addressed viral immune evasion. PA-X is a fusion product expressed by numerous influenza viruses, and an avian-origin H5N1 PA-X was recently shown to reduce influenza virus-induced nuclear localization and activity of NF-κB p65 in Madin Darby bovine kidney cells *in vitro* (J. Hu et al. 2020). It is possible that this represents a novel mechanism of AIV immune evasion that operates in mammalian cells, but it remains to be tested in avian cells, or *in vivo*.

In the past five years, the importance of the microbiota in shaping immune responses has come to light. Whilst earlier studies focused on mice, several more relevant to AI have been conducted, all confirming the role of the mucosal microbiota. For example, Figueroa *et al*. treated ducks with broad spectrum antibiotics to deplete both respiratory and intestinal microbiota and compared their responses to HPAI H5N9 with those of untreated ducks: the researchers found that antibiotic treatment had profound effects on gut immunity, leading to impaired RIG-I and type I IFN-induced gene expression, reduced IgA levels and increased virus shedding in faeces (Figueroa et al. 2020). In chicks, antibiotic treatment similarly led to increased cloacal virus shedding with reduced production of type I IFNs and IL-22 in the lung and intestine during infection with H9N2 LPAI, which could be partially prevented by post-antibiotic pre-infection administration of pro-biotics (Alexander Yitbarek, Taha-Abdelaziz, et al. 2018). Despite profound effects on innate mucosal immunity, the same group showed that antibiotic-treated specific-pathogen-free (SPF) chicks exhibited comparable antibody responses to their untreated counterparts following H9N2 infection (A. Yitbarek et al. 2018), suggestive of at least a partial decoupling between the microbiota-dependent and -independent mechanisms required for innate and adaptive responses to AI infection in this setting. Similar studies in mice identified microbially-derived desaminotyrosine as a potential candidate molecule mediating the pro-interferon effects of the gut microbiota (Steed et al. 2017) but its relevance in avian species remains to be tested.

Furthermore, there is some evidence that influenza virus infection itself disrupts the intestinal microbiome: Yitbarek at al. found that SPF chicks infected with LPAI H9N2 exhibited disrupted gut microbiota homeostasis, which the researchers speculated could assist the establishment of the infection (Alexander Yitbarek, Weese, et al. 2018). In agreement with the concept of an influenza-host-microbiome relationship, a large study characterising the faecal microbiota of five species of wild

ducks with and without IAV infection identified marked species-specific differences in the presence of IAV infection (Hird et al. 2018): thus, IAV infection is frequently linked with differences in the intestinal microbiota, but the exact nature of those differences varies by duck species. These studies reinforce the importance of moving away from widespread antibiotic (mis)use in poultry farming and also suggest that interventions aiming to shape a pro-immune/anti-viral microbiota could form a key part of an effective AI control strategy in the future. Further research is needed in the field to understand how specific features of the microbiota are associated with protection from AI and how effective interventions targeting the microbiota might be under real-world conditions.

Adaptive Immunity to Avian Influenza

The identification of cytotoxic T lymphocyte (CTL) epitopes within AI that are recognized by the chicken immune system has been lacking until recently. Zhang *et al*. identified NP67-74 from H5N1 (A/Goose/Gongdong/1/96) as a potent CTL epitope polypeptide within AI that was bound by MHC class I haplotype BF2*15 in chickens (Weijun Zhang et al. 2016). Importantly, following DNA vaccination with a plasmid encoding NP, the NP67-74 polypeptide stimulated strong recall CTL and IFNγ responses *in vitro* demonstrating its dominance in these chickens *in vivo*. The relevance of this epitope during challenge experiments is currently unclear.

A persistent question is how natural infection with LPAI can induce long-lasting immunity to re-infection with HPAI, while many vaccines fail to achieve the same feat. A recent study found increased expression of cytotoxicity-related genes including TNFα, NK lysin and granzyme A in lung cells from SPF chickens infected with low pathogenicity H9N2, whilst vaccination with inactivated H9N2 did not induce the same changes (Dai et al. 2021). Similarly during LPAI infection, another group uncovered marked differences in cellular response between two chicken breeds that exhibit relatively high- and low- resistance to influenza virus infection: Aston et al. correlated the relatively higher B and T cell frequencies seen in the trachea of the resistant Fayoumi breed after challenge with the low viral titre in swab samples, while the more susceptible Leghorn chickens failed to increase B and T cell frequencies in trachea and exhibited higher viral titres (Aston et al. 2021).

The role of adaptive immunity in determining both homo- and hetero-subtypic immunity to IAV in re-infection settings has also begun to be dissected. Latorre-Margalef *et al.* experimentally infected mallard ducks with LPAI H3N8 and then, five weeks later, groups of these birds were challenged with either the same virus or one of four heterologous IAV subtypes (Latorre-Margalef, Brown, et al.

2016a). After primary infection with H3N8, ducks exhibited partial cross-protection that was related to the genetic relatedness between the HA of the primary and secondary viruses (Latorre-Margalef, Brown, et al. 2016b). Taking these findings further, Wille *et al.* immunized laboratory-reared ducks with inactivated H3N8 and H6N2 IAV subtypes and exposed them to naturally-infected wild ducks which gave the opportunity to study multiple infections and co-infections under field conditions (Wille et al. 2017a). They found that H3 vaccination did induce H3-specific serum antibodies, but that ducks were not protected from H3 infection, or indeed from subsequent H3 re-infection: H6 viruses were not detected during the natural challenge experiment and so the protective efficacy of the vaccine could not be assessed (Wille et al. 2017b). Although there was not evidence of heterosubtypic protection in this study, the authors noted that the group's previous wild-duck monitoring study suggested that secondary and tertiary heterosubtypic infections are shorter and less intense (Avril et al. 2016).

Advances in Understanding of Avian Immunology

Given the importance of the Type-I IFN response in avian influenza, it is noteworthy that a study by Glotis *et al*. defined the global transcriptional response of chick embryonic fibroblasts to avian IFNα *in vitro* (Giotis et al. 2016). The researchers identified 128 genes whose expression was significantly differentially regulated in cells in response to treatment. These data may be a useful resource for future studies into the downstream effects of IFNα signalling in chicken cells, and for comparison with gene expression patterns induced by influenza viruses.

Another significant development is the finding that the cellular immune systems of chicks is functionally immature until approximately three weeks of age. While some studies have not seen any association between age and mortality from HPAI infection in commercial poultry as young as five weeks of age (Bertran et al. 2016), a recent study found that infection with an HPAI mutant that was survived by adult chickens was fatal in young chicks, but that their susceptibility could be prevented by the transfer of adult lymphocytes (Schmiedeke et al. 2020). These findings have potential implications for the vaccination program of young chickens, as they highlight a developmental window in which effective cellular immunity is unlikely to be stimulated and the chicks remain highly susceptible to infection. It is possible, however, that the presence of maternal antibodies could compensate for a chick's inability to mount effective responses during this time, with evidence that maternally-derived vaccine-induced antibodies to H7N3 HPAI can protect chicks from challenge for

the duration of this 3-week window of vulnerability (Cardenas-Garcia et al. 2019). The interaction between cell-mediated, antibody-mediated and innate responses in determining susceptibility versus resistance is yet to be explored.

New Tools for Immunological Studies of Avian Influenza

Several immunological techniques and tools have been advanced in recent years and could be applied to study the immune response of avian species to AIV in more detail. Two studies report improved methods for multi-parameter flow cytometry of chicken immune cells (Hofmann and Schmucker 2021; Hao et al. 2020), while another study demonstrated the utility of IFNγ ELISpot for detection of virus-specific CD8 T cell responses to AIV infection (Ruiz-Hernandez et al. 2015). The authors noted that coupling this technology with the use of recombinant viruses could assist in the definition of antigen specificity and CD8 T cell epitopes, which could aid rational vaccine design in future.

Another important advance for *ex vivo* immunological study is the recent definition of a working protocol for generation and use of precision cut lung slices from adult chicken lungs, which can be maintained in culture for around 40 days (Bryson et al. 2020). This approach has major advantages for virological and immunological studies as it retains the organisation and cellular diversity of the *in vivo* tissue and allows for extended monitoring: Bryson *et al.* also demonstrated the potential utility of lung slices for the study of LPAI (Bryson et al. 2020), and it will be interesting to see what knowledge can be gained from further exploration of this approach.

An important aspect of effective experimental design is validation of the models being used. Recent work by van Dijk *et al.* confirmed that laboratory mallards are an appropriate model for their wild counterparts (van Dijk et al. 2020), giving greater confidence that conclusions generated in laboratory duck populations may well be applicable to free-living birds.

Swine Influenza Immunology

Host Immunogenetics

Although host genetics are likely to play a key role in immunity to SIV vaccines and infection, few studies have aimed to assess specific links between these factors. Zanella *et al*. conducted a genome-wide association study in 103 pigs from a cross-bred population vaccinated with an H1N1 WIV vaccine (Zanella et al. 2015a). The authors identified a total of seven genomic loci that were linked to levels of anti-NP or anti-HA antibody post-vaccination: among these loci were genes involved in down-regulation of the Th17 response and mediating signal transduction in T cells (Zanella et al. 2015b). The genes identified in this study are valid targets for further investigation and may have relevance to breeding strategies for enhanced disease resistance. It remains to be established whether naturally more influenza-resistant swine vary at these loci compared to commercial pig breeds, and whether the same loci are involved in responses to different types of vaccines/adjuvants/immunization routes.

Innate Immunity to Swine Influenza

The innate immune response in the lung represents one of the first lines of defence following exposure to influenza virus. In the first days post-infection, swine infected with H1N2 exhibit significant increases in expression of genes encoding type I IFNs, as well as RIG-I, MDA5 and TLRs 3 and 7; alongside, the transcription of pro-inflammatory cytokines including IL-1 and IL-6, and of the anti-inflammatory cytokine IL-10 (Brogaard et al. 2018). Brogaard *et al*. also uncovered a 400-fold increase in expression levels of pulmonary IFN-λ (Brogaard et al. 2018), a type III interferon that is important in mediating the murine defence against IAV (Galani et al. 2017), but is relatively unstudied in swine. These data confirm and extend findings from an earlier study that showed that porcine alveolar macrophages infected with H3N2 SIV *ex vivo* significantly increased expression of TLR-3, RIG-I and MDA5 from 4 hours post-infection; by 8 hours, the researchers also detected significant changes in MyD88, MAVS, IRF-3 and IRF-7 mRNA expression levels, as well as increased TNF-α, IL-1β, IFN-α and IFN-β mRNA and protein levels (Jinqiu Zhang et al. 2015).

The importance of type I IFNs in response to influenza virus infection is well established. At the cellular level a role has now been identified for interferon-induced protein with tetratricopeptide repeats (IFIT)3, whose expression is increased in immortalised porcine alveolar macrophages during *in vitro* infection (Yongtao Li et al. 2015) The researchers found that IFIT3 overexpression inhibited SIV

replication and enhanced IFNβ promotor activity by targeting the mitochondrial antiviral signalling protein, MAVS (Yongtao Li et al. 2015). IFNβ production during IAV infection may also be moderated by the NLRP3 inflammasome – a key molecular platform contributing to pro-inflammatory cytokine production during viral infection (C. Zhao and Zhao 2020). Park *et al*. infected porcine alveolar macrophages with SIV H1N1 and H3N2 *ex vivo* and found that production of the pro-inflammatory cytokine IL-1β in response to viral replication was NLRP3-dependent (H.-S. Park et al. 2018). In a subsequent study, the same group revealed that SIV infection in this system led to phosphorylation of DRP1 and its translocation to the mitochondrial membrane, leading to mitochondrial fission, production of reactive oxygen species and consequent enhancement of IL-1β production by the NLRP3 inflammasome (H. S. Park et al. 2018).

There is significant evidence in humans and mice of the importance of lung and gut microbiota in resistance to influenza virus infection (Yuan et al. 2020). However, the possible immune-modulating effects of commensal bacteria in pigs are only beginning to be investigated (as reviewed in (Patil, Gooneratne, and Ju 2020)), and their interaction with the response to influenza infection is unknown.

According to the importance of the type I interferon response in combatting influenza virus infection, antagonising the production of antiviral products by host cells is an important mechanism of influenza immune evasion. Early work identified SIV NS1 protein as a potent IFNα/β antagonist and *in vivo* virulence factor (Solórzano et al. 2005). More recently, studies in a canine cell line showed that the S42 residue within the NS1 protein of swine H1N1 was able to block the action of IRF3 and thereby inhibit the production of IFNα and β: a mutation at this position led to reduced virus growth *in vitro* (J. Cheng et al. 2018). Better understanding of such mechanisms of immune evasion may support the development of effective anti-viral interventions targeting the disrupted pathways to reinstate full anti-viral function.

Under field conditions, swine may be infected by more than one respiratory virus at a time, and an open question had been how this affects the magnitude and type of innate immune response. A recent study showed that there was no significant difference between the lung cytokine profile or the amount of cytokines produced at days 2 or 4 post-infection in piglets intra-nasally inoculated with H1N1 alone, porcine reproductive and respiratory syndrome virus alone, or both viruses together (Turlewicz-Podbielska, Czyżewska-Dors, and Pomorska-Mól 2021). By contrast, when piglets were co-infected with *Actinobacillus pleuropneumoniae* and SIV, lung concentrations of IL-1β, IL-8, and IFN-α were significantly higher than in piglets infected with either pathogen alone ((Czyzewska-Dors et al. 2017). Different again, when porcine lung slices were infected *ex vivo* with SIV and porcine respiratory

coronavirus, Krimmling et al. uncovered evidence of viral interference, leading to reduced titres of both pathogens(Krimmling and Schwegmann-Weßels 2017). Therefore, it seems likely that whether and how the porcine lung immune response is modified during co-infection likely depends on the identity of the co-infecting pathogen, and this may well have to be assessed on a "case-by-case" basis.

Adaptive Immunity to Swine Influenza

To develop subtype cross-protective vaccines against SIV, there is a need to define conserved antigenic epitopes. A recent study immunized groups of inbred pigs with different combinations of UV-inactivated H1N1, a commercial preparation of swine H3N2, H1N1 and H1N2 subtypes, and a live recombinant IAV bearing the H5 HA with the rest of the proteins from human IAV A/PR/8/34, then tested their T-cell restimulation responses to a set of peptides *ex vivo* (Baratelli et al. 2020). The authors identified $NP_{406-416}$ as a putative swine leucocyte antigen (SLA) -II cross reactive T-cell epitope that warrants further investigation for incorporation in rationale vaccine designs; however, the power of these findings was limited by the small sample size and further experiments will be required to firmly establish the validity of the epitope. Computer modelling may also be helpful in this regard (for more information see here).

In the case of CTL epitopes, there is encouraging *in vitro* evidence that several SIV H1N1 peptides may bind SLA-3*hs0202 and the most common human MHC allele, HLA-A*0201 (Fan et al. 2018). Such studies support the idea that it might be possible to generate multi-species vaccines against some types of IAV in the future. While such targeted epitope binding and prediction approaches are undoubtedly useful, they are limited to assaying recall responses that test for reactivity to those peptide epitopes. Another approach is to ask which epitopes are presented naturally by infected cells, and then to identify them. Lamont *et al.* used primary porcine airway epithelial cells infected with SIV H3N2 *ex vivo* and incubated them for 24 hours before eluting SLA-I-bound peptides for identification by mass spectrometry (Lamont et al. 2018). They identified peptides from across M1 and NP, with exciting evidence of SLA-I presentation of peptides within the region of M1 that is conserved across multiple SIV strains (Lamont et al. 2018), which could be incorporated into CTL-targeted cross-protective vaccine strategies. Similarly, in a study combining epitope prediction and detection of peptide-specific specific T cells from the blood of immunised animals, Baratelli *et al*. detected recognition of T-cell epitopes within M1 and NA proteins that were conserved between avian-like SIV H1N1 and H1N1 of the 2009 pandemic lineage (Baratelli et al. 2017).

The larger scale T-cell response to SIV infection of pigs has also begun to be described in more detail. Complementing their earlier studies on circulating T-cell populations in SIV-infected swine (Talker et al. 2015), Talker *et al.* went on to characterise cellular immunity in the lungs of piglets infected with H1N2 SIV. Following inoculation, the researchers revealed the emergence of virus-specific IFNγ-producing $CD4^+$ and $CD8^+$ T cells in the lung from as early as 4 days post-infection, followed by an influx of proliferating $CD8^+$ T cells from 6 days post-infection that was accompanied by the onset of high levels of antibody in serum (Talker et al. 2016). Lung samples taken at 6 weeks post-infection contained both $CD4^+$ and $CD8^+$ memory T cells, which exhibited heterologous reactivity against H1N1 and H3N2 SIVs when restimulated *ex vivo* (Talker et al. 2016), again supportive of the development of T-cell-mediated cross-protective vaccines for SIV.

In mice, tissue-resident memory cells T cells in the lung have been found to be important for cross-protection following intra-nasal immunization with the LAIV human influenza vaccine, FluMist (Zens, Chen, and Farber 2016). Initial evidence suggests that a comparable cell population may be elicited in the lungs of pigs following aerosol administration of the single-cycle LAIV S-FLU; here, the presence of tissue-resident memory cells was associated with reduced pathology following hetero-subtypic challenge (Holzer et al. 2018). How these observations fit in the overall picture of immunity to SIV during infection or after vaccination remains to be elucidated.

The effects of maternal antibodies on the immune responses of pigs to SIV are also gradually becoming clearer. A recent study by Pardo *et al*. highlighted the potential for MDA to reduce SIV prevalence and increase the time before first SIV infection in weaning piglets in the field; however, they also noted the high heterogeneity of MDA levels amongst piglet populations from similarly-vaccinated sows (Pardo et al. 2019). Variability of MDA levels in piglets does not appear to relate to differences in antibody titres in sows (Sisteré-Oró et al. 2019a); thus is would be interesting to establish the basis for the heterogeneity among piglets. Such knowledge could be useful in the design of more effective vaccination strategies aiming to induce reliable protection of piglets until the optimal timepoint of first vaccination.

A key point regarding MDA is whether they affect the likelihood of transmission of SIV under different circumstances. Deblanc *et al*. recently showed that SPF piglets bearing MDA to H1N1, H3N2 and H1N2 were resistant to clinical disease following H1N1 challenge, and that this resistance waned as did the levels of MDA (Deblanc et al. 2018), which have been shown to persist for 2-to-3 months after birth (Cador, Hervé, et al. 2016b). From five weeks of age, all piglets were able to mount responses to H1N1

challenge that protected them from subsequent reinfection, regardless of the presence of MDA; yet intriguingly, while levels of viral genomes were comparable in nasal swab supernatants from MDA and non-MDA piglets challenged at this age, the *in vitro* infectivity of the virus in MDA-bearing piglets was significantly lower (Deblanc et al. 2018). Despite this, previous studies have shown that MDA-bearing piglets *are* capable of transmitting infection to MDA- positive and negative piglets, albeit with a significantly lower R number compared to piglets lacking MDA (Cador, Hervé, et al. 2016b), and, in one case, that detection of live virus in air samples from challenged piglets was dependent on the presence of heterologous MDA and did not occur when the MDA matched the challenge strain (Corzo et al. 2014). In contrast, a recent field study documented frequent infection of piglets within the first five weeks of life, despite the presence of MDA (Ryt-Hansen, Larsen, Kristensen, Krog, Wacheck, et al. 2019), although the levels and specificity of the MDA in piglets was not assessed.

New Tools for Immunological Studies of Swine Influenza

Knowledge of the role of T cells following vaccination or during infection with SIV is limited, in part due to a lack of tools with which to study porcine responses. Work by Tungatt et al. represents an important step forwards: using the Babraham inbred pig they developed a comprehensive toolset for the study of $CD8^+$ T cell response and showed its utility for characterising the response to SIV immunisation and infection (Tungatt et al. 2018). The authors defined the structures of two SLA-I molecules, identified the primary anchor points for immunodominant SIV T cell epitopes within their grooves and characterised several novel peptide sequences from NA that are recognized by SIV-specific CD8 T cells from immunized or infected pigs. They also generated a long-term pig T cell clone for *in vitro* culture and further studies.

Holzer *et al.* recently reported the generation of the first porcine monoclonal antibodies recognizing H1N1 HA, which will enable enhanced monitoring of antigenic drift of IAV as well as representing a useful tool for immunological studies of influenza viruses that can infect both swine and humans (Holzer et al. 2021).

Monitoring the immune response of pigs in real-time is critical for our understanding of their response to infection and vaccination. Yen et al. have reported an improved minimally-invasive technique for cannulation of the thoracic duct, allowing collection of lymph (and therefore immune cells) draining from respiratory tissues without marked tissue damage and the associated inflammation this causes (Yen and Davies 2016).

Equine Influenza Immunology

Literature searching retrieved no studies investigating the immune response to equine influenza in horses in the past six years.

Companion Animal Influenza Immunology

CIV varies markedly in pathogenicity in dogs, but the reasons for this have been unclear. A study comparing microRNA expression in lungs from beagles infected with high (H5N1 AI) and low (H3N2 AI) pathogenesis CIV revealed marked differences, suggestive of modulation of metabolic pathways and inhibition of innate immunity in the high-pathogenesis group (Y. Zheng et al. 2018).

Canine STING is expressed abundantly in the spleen and lung during the steady state. *In vitro* overexpression of STING resulted in induced IFN, increased ISG15 and viperin expression, and inhibited H3N2 canine influenza virus replication (Y. Zhang et al. 2017). However, Zhang et al. found that STING was not necessary for the IFN-β response to poly(dA:dT), poly(I:C), or Sendai virus. The precise role of STING in anti-viral immunity and specifically influenza virus immunity, remains to be defined.

Ongoing research

In the UK, researchers at the University of Nottingham are exploring the interactions of IAV and IDV proteins with host immune responses (e.g. the role of host protease in IDV HEF protein activation and interactions between the NS1 protein and swine cell-signalling pathways), and an upcoming study here will focus on the importance of the phosphoinositide 3-kinase pathway in modulating IAV replication in chickens and ducks. At the French ANSES, scientists are currently studying training of porcine alveolar macrophages by SIV and its possible impact on innate immune responses towards heterologous viral superinfections. Meanwhile in the USA, researchers at the University of Minnesota are conducting an international collaboration with UK scientists to evaluate how IBDV-related immunosuppression influences AIV transmission and evolution in wild and domestic birds. Resulting data on infectious dose, transmission rates, R_0, etc. will be used to generate transmission and surveillance models.

At the Friedrich-Loeffler-Institut, scientists are examining the spillover potential of bat IAV-like viruses, specifically relating to their host cell immunological interactions (e.g. cellular factors associated with cell entry/endosomal release and innate immune proteins that act as restriction factors), and at the Norwegian Veterinary Institute, research continues into the impact of avian endothelial cell programs on the activation or blunting of antiviral responses. Conversion from an LPAI to an HPAI phenotype involves acquisition of endothelial tropism, and blunting of this tropism can ameliorate disease – these studies may therefore enhance our understanding of the immunological underpinnings of HPAI virulence and resistance in various bird populations.

Researchers at the Royal Veterinary College at the University of London and OIE/FAO International Reference Laboratory are currently engaged in a collaboration with the American National Institute of Allergy and Infectious Diseases – the Centers of Excellence for Influenza Research and Response program (CEIRR) – that conducts wide-ranging surveillance and epidemiological studies including research into immune responses against vaccination and infection. At Ghent University in Belgium, meanwhile, the DELTA-FLU five-year project encompasses a wide array of interdisciplinary aims relevant to virology, environmental epidemiology, and immunology – specific to the latter, researchers are currently studying the effect of flock immunity against AIV on early detection and genetic drift.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of animal influenza immunology should be considered priorities for future research:

- *The application of immunogenetic study findings to generating more resistant commercial poultry species*
- *Immunogenetic studies in swine*
- *Information on a possible carrier state in some species or immune-suppressed individuals*
- *Translation of recent findings on possible innate immune determinants of susceptibility in avian species into generation of more resistant breeds*
- *Definition of the role of specific cytokines in reducing viral shedding and increasing resistance to infection*
- *How dietary/other interventions might be targeted towards eliciting changes in the microbiota that support immunity to influenza and/or improve responses to vaccines*

- *The cellular immune response of chickens to HPAI and its significance during infection*
- *Widespread validation of the findings of laboratory studies under field/near-field conditions*
- *Characterisation of the post-infectious adaptive immune response that determines protection from reinfection with homologous or heterologous strains*
- *Understanding the role of factors such as pregnancy and obesity on the susceptibility of breeding sows to SIV infection*

Controlling the Disease

Surveillance

Current methods for veterinary influenza surveillance include population monitoring (e.g. swabs from animals in poultry and swine farms), bioaerosol testing, environmental sampling (e.g. water samples from migratory bird resting sites), and targeted active surveillance (Schar et al. 2019; Munyua et al. 2019). Data from these efforts allow public health officials and researchers to monitor animal production networks for signs of disease, quickly respond to changing transmission patterns during ongoing outbreaks, and keep tabs on emerging flu strains that may be circulating within wild bird populations. Surveillance is an expensive endeavour that requires integration into existing government health networks and cooperation from landowners, poultry farmers, live bird market workers, and others within the animal production pipeline. Flint *et al.*, for instance, argue that active surveillance should be targeted to areas where biosecurity measures can be most efficiently implemented to reduce economic loss (e.g. regions of high domestic poultry as opposed to watersheds/wild duck populations) (Flint et al. 2015). Surveillance systems must also be tied closely to research advancements in virology and epidemiology – the identification or genome sequencing of new reassortant strains, for instance – to ensure that limited resources are focused on the geographical regions, animal species, and influenza strains most relevant to current epidemiological patterns (Alkhamis, Li, and Torremorell 2020).

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps in animal influenza surveillance in 2014/15:

- *systematic approaches for analysing and reporting viral genome sequences*
- *effective systems for monitoring influenza at the human/animal interface*
- *inclusion of epidemiological, environmental, and socioeconomic data into comprehensive viral prediction systems*
- *development of efficient sampling methods for rapid detection of emerging strains*
- *assessment of high-risk areas to target limited surveillance resources for maximum impact*
- *integration of diagnostic and surveillance data systems*

Literature review

As computational modelling capabilities continue to gain power, much recent attention has focused on applying these resources to new, efficient methods of influenza surveillance. Pereira *et al.* demonstrated a "fireworks" model, where the trajectory of outbreaks is viewed as a series of short-range local transmissions and long-range propagation events, for describing the spread of HPAI H5N1 AIV in wild birds in Europe. La Sala *et al.*, meanwhile, took a more combinatorial approach, integrating expert opinion, multicriteria decision analysis, and ecological niche modelling to calculate a spatially explicit risk index for identifying high-risk sites for LPAI transmission from wild birds. Unsurprisingly, they found this risk to be significantly higher near commercial poultry farms (La Sala et al. 2019). Expert opinion has proven a useful addition to integrated surveillance approaches – Paul and colleagues, for instance, studied HPAI H5N1 risk in Thailand and found that predictive power was substantially higher for risk maps drawn using local experts' opinions than those produced from literature review (Paul et al. 2016). Other studies have focused on novel applications of existing datasets, with Belkhiria *et al.* testing the use of previous LPAI suitability maps for prediction of areas at high risk for HPAI outbreaks. Working within the context of the North American HPAI H5Nx outbreaks of 2014-2015, the authors applied species distribution modelling to map HPAI suitability over each of the four North American migratory flyways, finding that 89% of HPAI outbreaks occurred in regions already known to be highly suitable for LPAI (Belkhiria, Alkhamis, and Martínez-López 2016).

Increasing computational capabilities are also reflected in the growing importance of internet-based databases as a source of integration between surveillance and other areas of influenza research. Recent developments have focused on the collaborative potential of such databases. Peyre *et al.*, for example, describe the EVA surveillance evaluation tool, a support network for integrating technical, process, and socioeconomic factors when evaluating existing surveillance systems. The EVA tool includes generalized evaluation work plans, a web interface for generating custom evaluation plan designs, and a Wiki-style classroom for providing theory education (Peyre et al. 2019). Other web-based resources focus on information mining and automated classification; these include the PADI-web, a French web platform that collects, processes, and extracts relevant information on disease outbreaks from Google News (Arsevska et al. 2018). Such informal, event-based surveillance reduces the required manpower and allows automated discovery of epizootic disease emergence, dynamics of enzootic diseases, and the emergence of non-notifiable diseases (Arsevska et al. 2018).

These computational networks rely ultimately on the efficient collection of frontline data, and novel methods for collecting such data remain one of the most important areas of influenza surveillance

research. Anderson and colleagues tested bioaerosol sampling as a method for SIV surveillance in five Guangdong province swine farms during the 2014 summer and fall/winter seasons, finding it to be a statistically significant predictor of SIV-positive pig oral secretions and environmental swab samples. Relatedly, Reeves and colleagues described a proof-of-concept for testing environmental persistence of AIV shed from waterfowl under ambient conditions. They inoculated filtered pond water with avian faecal samples, then placed these samples in sealed vials and submerged within Alaska wetland terrain for up to 132 days. Samples were subsequently tested via rRT-PCR and virus isolation, with virus isolation possible for up to 118 days (Reeves et al. 2020). Depending on their scope of detection, surveillance systems may also be combined with active deterrence to create an automated network designed to limit AIV transmission at high-risk sites. Atzeni *et al.* describe one such endeavour, reporting a machine vision-based surveillance system that detects and deters target wildlife (specifically ducks, the natural reservoir of HPAI) from poultry farms in Australia. They tested proof-of-concept for a solar-powered, Wi-Fi-connected system using a surveillance camera and custom image-processing/classifying software to automatically detect and recognize birds of target species, tying this system to digital deterrence methods (e.g. noise- or light-based) to produce a closed-loop automated detect-alert-deter system (Atzeni et al. 2020). Such rapid detection biosensors can also be combined with data on migratory flyway patterns to efficiently produce predictive risk maps (Astill, Fraser, et al. 2018), generating more data for wide-scale surveillance efforts.

Frontline reporting of identifiable diseases to public health authorities remains one of the most important avenues of influenza surveillance, bringing with it many challenges relating to socioeconomic factors. Kurscheid *et al.*, for instance, surveyed LPM poultry traders in Bali and Lombok, Indonesia, finding that two thirds of respondents were not willing to report sudden or suspicious bird deaths to the government (Johanna Kurscheid et al. 2015). Such willingness did not positively correlate with better education, suggesting a need for increased incentives for disease reporting. Such incentive networks may be more difficult to deploy in resource-limited countries where local trade and development imperatives are likely to outweigh disease control and public health concerns (Mwacalimba and Green 2015). Minimally-invasive surveillance methods may also play an important role in generating surveillance data without interfering in day-to-day economic necessities – Choi *et al.*, for instance, trialed the use of telemetry (via small GPS transmitters) to record domestic duck movement and poultry transport from source farms and through market chains in China, providing direct information on poultry market network connectivity (C. Y. Choi et al. 2016).

Ongoing research

In France, ANSES, which hosts the National Reference Laboratory for Swine Influenza, established RESAVIP, a public-private national surveillance network for influenza virus in pigs. The passive surveillance carried out within this network makes it possible to define the genetic diversity and geographical distribution of swIAVs across France. ANSES also contributes to specific surveillance programmes in certain farms/regions, as well as conducting occasional surveys of wild boars. Researchers there are currently working on surveillance of influenza virus types B, C and D, which are less common and much less pathogenic in pigs than IAV.

Scientists at the Friedrich-Loeffler-Institut, in collaboration with the University of Freiburg's Institute of Virology, are conducting surveillance on workers and animals in large commercial swine holdings, conducting surveillance for potential zoonoses and associated determinants. Similar studies are underway at the University of Minnesota, where researchers are actively surveying swine farms during peak influenza season to evaluate worker influenza positivity rates, use of sick leave, and risk factors for zoonotic transmission. At Erasmus MC Viroscience, researchers conduct surveillance of wild birds to elucidate LPAI ecology and routes of HPAI incursion via migratory patterns. Scientists at the National Institute of Agriculture Technology, meanwhile, are involved in similar surveillance of AIV in wild birds in the southern cone of South America and also conduct surveillance in swine populations. The Nigerian National Veterinary Research Institute is involved in multiple surveillance programs as well, conducting targeted AIV surveillance in high-risk areas (e.g. wetlands and LPMs) and multispecies monitoring in swine, equines, and canines.

Scientists at the University of Minnesota are also involved in surveillance methods comparison, identifying udder wipes from lactating sows as a novel, convenient, inexpensive test that maintains high sensitivity for monitoring SIV in litters prior to weaning. In the U of M College of Veterinary Science, meanwhile, scientists are conducting surveillance of wild gull populations, which remain understudied despite their frequent interactions with domestic poultry farms. Researchers in the UK at the Animal and Plant Health Agency are heavily involved in international influenza monitoring and surveillance, rapidly characterising viral evolution and strain emergence and linking together international efforts to WHO collaborating centres.

In the Russian Federation, meanwhile, work continues at the Federal Research Center for Virology and Microbiology to survey and genotype Russian AIV isolates e.g. by conducting primary screening of samples from wild birds, isolating viruses from chicken embryos and sequencing, and making information available in the FRCVM collection of microorganisms. The National Institute of Animal

Health, SIV surveillance is conducted on Japanese swine populations, while the National Agricultural Technology Institute carries out similar monitoring on equine influenza in Argentina. Ghent University in Belgium participates in OIE/FAO's OFFLU network, performing SIV surveillance and providing data to strengthen and coordinate worldwide surveillance efforts and methods.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of animal influenza surveillance should be considered priorities for future research:

- *Standardization of international surveillance programs for avian and swine influenza*
- *Improvement of surveillance systems in wild birds*
- *Early detection of zoonotic (or potentially zoonotic) influenza strains for pandemic prevention*
- *Early detection systems for influenza A virus in turkeys and pigs*
- *Monitoring of point-of-entry into high-risk areas (introduction of HPAI from Asia/Europe to Nigeria) via migratory waterfowl*
- *New methods for anticipating/detecting potentially dangerous viral subtypes prior to major outbreak events*
- *Early-warning risk-based surveillance systems designed for emerging events in both wild and domestic hosts*
- *Surveillance for swine influenza in pig populations worldwide, including antigenic and genetic characterization of contemporary circulating strains*
- *Applied research on novel/highly relevant viruses with heightened outbreak risk*

Diagnostics

Accurate and timely diagnosis of viral influenza infection in animal species is vital for a number of reasons. Infection has direct health implications for affected individuals, so limiting viral spread promotes animal welfare. Outbreaks of influenza can also have drastic consequences for both infected and uninfected animals and economic impacts on farmers, as the need to prevent the spread of IAVs, and especially those with zoonotic potential, can necessitate the culling of very large numbers of animals on affected farms.

The particular evolutionary dynamics of IAV present specific challenges to diagnosis. Many subtypes and strains of IAV currently exist, and they differ considerably in host range and pathogenicity. In addition, the viral genome is prone to reassortment and mutation, leading to the emergence of new variants. The HI assay is well characterised, as the traditional standard method for IAV serotyping, but it is not suited to pen-side use, high throughput or simultaneous testing for multiple viral subtypes and/or other pathogens. HI generally requires propagation of virus in cells or eggs prior to testing, which takes time and can lead to viral mutation, affecting the accuracy of the HI results. Although the introduction of the ELISA and PCR methodologies to IAV diagnostics has demonstrated great potential to facilitate viral identification, further refinements are necessary to optimise the use of these techniques.

Current recommendations

The *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019* contains information on the current OIE recommendations for diagnosis of avian, equine and swine influenza infections. The *Terrestrial Manual* also contains information on management of diagnostic laboratories, collection, storage and transport of specimens, and validation of diagnostic tests.

For the purposes of notification and control, the OIE defines avian influenza as infection of poultry caused by any high-pathogenicity IAV, or by low-pathogenicity H5 or H7 IAV. The *Terrestrial Manual* lists virus isolation, antigen detection and RT-PCR as methods for AIV identification, and agar-gel immunodiffusion (AGID), HI and ELISA as methods for detection of immune responses. Although virus isolation is described as the 'gold standard' for AIV identification, it is acknowledged to be labour-intensive and relatively slow, and therefore recommended for use primarily for diagnosis of index

cases and for isolation of virus for further analysis. Virus isolation typically proceeds by inoculation of SPF embryonated chicken eggs, incubation for 2–7 days, chilling and recovery of allantoic fluid, which is tested for the presence of AIV by methods such as haemagglutination, AGID, ELISA, HI, NA inhibition (NI) and rRT-PCR. AGID is used for detection of NP and M antigens in concentrated allantoic fluid or extracts of chorioallantoic membranes. Adding these samples and appropriate positive antisera to wells in agar plates enables diffusion of antigens and antibodies into the agar, where interaction is demonstrated by formation of a visible line of precipitate. Antigen-capture ELISAs are alternatives to AGID. Haemagglutination does not distinguish between the presence of AIV or an avian paramyxovirus, but the presence of NDV can be evaluated by HI testing. RT-PCR and rRT-PCR using primers specific for conserved sequences of *NP* or *M* genes are suitable methods for identification of the presence of AIV, and H5/H7 subtypes can also be identified through the use of appropriate subtype-specific primers. Subtyping can also be achieved by antigenic characterization in HI and NI tests, using monospecific antisera to subtype-specific HA and NA proteins, or polyclonal antisera to specific intact AIVs. Pathogenicity of isolated AIV can be determined directly, by IVPI assay or similar intravenous inoculation method in susceptible chickens. In addition, for H5 or H7 LPAIVs, the sequence of the HA proteolytic cleavage site should be determined, as similarity with known HPAIV sequences is sufficient to result in classification as HPAIV. In addition to the sequencing of individual genes for subtyping and pathotyping, complete genomic sequencing is important for monitoring of viral reassortment and evolution.

For H3N8 EIV, the *Terrestrial Manual* lists the methods of agent identification as virus isolation, rRT-PCR, RAD and antigen-capture ELISA, with detection of immune responses by HI, single radial haemolysis (SRH) and ELISA. Virus isolation is generally carried out by inoculation of embryonated hens' eggs, with identification of EIV in amniotic and allantoic fluids by haemagglutination assays. RT-PCR and ELISA can be used for diagnosis, or to select samples for virus isolation and further characterization. For EIV subtyping, HI can be carried out with antisera specific to H3N8 and H7N7 (which is not currently circulating). *HA* and *NA* gene sequences can also be determined by RT-PCR and sequencing. Serological tests for infection include HI (using virus/viral antigens for haemagglutination), SRH and ELISA. In SRH, test sera are added to wells in agarose containing viral antigens coupled to fixed red blood cells, along with guinea-pig complement. Diffusion of antibodies to EIV into the agarose results in cell lysis and a clear haemolytic zone.

For IAV in swine, the *Terrestrial Manual* lists the main methods of agent identification as virus isolation, HI and PCR (rRT-PCR and conventional PCR), with detection of immune responses by HI and

ELISA. Notably, swine IAV is a potential human pathogen, requiring appropriate biological safety measures. Virus isolation is by egg inoculation or cell culture, with haemagglutination to determine its presence. HI is used for antigenic typing of viral isolates, which can also be achieved by fluorescence-labelled-antibody staining of infected cells or tissue samples, immunohistochemistry, antigen-capture ELISA and PCR. Options for viral identification by RT-PCR include amplification of sequences corresponding to the *M* and *NP* genes, and a number of assays are also available for viral subtyping. Serological subtyping is carried out on paired serial serum samples by HI assay, or by ELISA.

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps and priorities in animal influenza diagnostics in 2014/15:

- *Early detection of virus with zoonotic and/or pandemic potential*
- *Development of tests for use in the field*
- *Development of DIVA tests*
- *Improvement of viral sampling and isolation*
- *Development of internal controls*
- *Development of rapid, cost-effective full-genome sequencing*
- *Improvement of systems for viral replication*
- *Subtype-specific testing capability, including antibody tests for sera*
- *Validation of existing diagnostics against novel isolates*
- *Development of rapid molecular tests that are robust to genetic mutations*
- *Development of transport media that stabilise the virus without refrigeration*

Literature review

Developments in testing for viral RNA, protein and antibody responses can relate to the general identification of IAV, to specific viral subtypes such as H1N1 and H9N2, or to individual genes or

antigens. Multiplex tests for simultaneous identification of multiple strains of IAV or for IAV and other pathogens are increasingly being developed. Developed assays match the host specificities of particular IAV subtypes and are designed for either lab-based or field-based application. Additional developments relate to the updating of PCR primers, assessment of storage media, and characterization of novel techniques for identification of infection.

There have been many more published developments in IAV diagnostics since 2015 than it is possible to describe here, and this literature review is therefore focussed less on improvements to existing methods, such as *M*-gene RT-PCR, ELISA and genome sequencing, and more on new applications of existing techniques, and the introduction of new techniques to IAV diagnostics. Notably, although these advances have been described in the following sections in relation to subtype specificity, it may also be helpful to compare them by methodology, as shown in Table 2.

Methodology	Lab/ field	Target	Host specificity	Subtype(s)	Reference
rRT-PCR	Lab	*HA* gene	Avian	H3	(Teng et al. 2015)
rRT-PCR	Lab	*HA* gene	Avian	H7 LPAIV/HPAIV	(Graaf, Beer, and Harder 2017)
rRT-PCR	Lab	*HA* gene	Avian	H9N2	(Saito et al. 2019)
Multiplex RT-PCR	Lab	*HA* gene	Avian	H1, H2, H3, H5, H6, H7, H9, H10	(M. Li et al. 2018)
Multiplex RT-PCR	Lab	*HA*, *M*, *F*, *N*	Avian	IAV (all, H5, H7, H9), NDV, IBV	(Q. Xiao et al. 2019)
Multiplex rRT-PCR	Lab	*HA*, *M* genes	Avian	IAV (all), H5 HPAIV/LPAIV	(Naguib et al. 2017)
Multiplex rRT-PCR	Lab	*HA* gene	Avian	H5 HPAIV	(Le et al. 2020)
Multiplex rRT-PCR	Lab	*NA* gene	Avian	N1–N9	(Z. Sun et al. 2017)
Multiplex rRT-PCR	Lab	*HA*, *NA*, *M* genes	Avian, swine	IAV (all), H1–H13, H16, N1–N9	(Hoffmann et al. 2016)
rRT-PCR	Lab	*HA*, *NA, M* genes	Swine	IAV (all), H1N1, H1N2, H3N2	(Bonin et al. 2018)
Multiplex rRT-PCR	Lab	*HA, NA, M*, *PB1*, *PB2*, *PA*, *NP*, *NS*	Swine	IAV (all), H1N1, H1N2, H3N2	(Goecke et al. 2018)
Multiplex RT-PCR	Lab	*HA* gene	Canine	H1N1, H3N2, H3N8	(C. Wang et al. 2017)
Multiplex rRT-PCR	Lab	*HA* gene	Equine	H3N8 (clade-specific)	(Brister et al. 2019)
Insulated isothermal RT-PCR	Field	*HA*, *NA* genes	Avian	H7	(Inui et al. 2019)
RT-LAMP/microarray	Lab	*HA* gene	Avian	H5N2, H6N1, H5 HPAIV	(L. C. Wang, Huang, and Chen 2016)
RT-LAMP	Field	*HA* gene	Avian	H10	(Luo et al. 2015)
RT-LAMP	Field	*M* gene	Avian	IAV (all)	(SHI et al. 2019)
RPA, lateral flow	Field	*HA*, *NA* genes	Avian	H7N9	(S. Ma et al. 2018)
RPA, lateral flow	Field	*HA* gene	Avian	H9N2	(Zeng Wang et al. 2019)
Multiplex RT-RPA	Lab	*NP*, *HA* genes	Avian	IAV (all), H5, H6, H7	(Tsai et al. 2020)
Sandwich ELISA	Lab	Virus	Avian	H3	(Luo et al. 2020)
Sandwich ELISA	Lab	Virus	Avian	H7	(Lingling Chen et al. 2019)

Sandwich ELISA	Lab	Virus	Avian	H9N2	(Ming et al. 2019)
ICS	Field	HA protein	Avian	H7N9	(Yeo et al. 2017)
ICS	Field	Virus	Avian	H7N9	(M. Xiao et al. 2019)
ICS	Field	Virus	Avian	H9N2	(Yeo et al. 2016)
Electrochemical immunosensor	Field	HA protein	Avian	H1N1, H5N1	(Veerapandian, Hunter, and Neethirajan 2016)
Antibody-conjugated vesicles	Field	HA protein	Avian	H5 AIV	(L. Jiang et al. 2015)
Surface plasmon resonance immunosensor	Field	Virus	Avian	H6N1	(X. Zhao et al. 2016)
Proximity ligation assay	Lab	Virus	Swine	H3N2	(Martin et al. 2017)
Magnetoresistance	Field	NP protein	Swine	IAV	(D. Su et al. 2019)
Rapid antigen detection	Field	Virus	Equine	H3N8	(Yamanaka et al. 2017)
Field-effect-transistor biosensor	Field	Virus	Avian/human	α-2,3-/α-2,6-linked sialic-acid-binding IAV	(Hideshima et al. 2019)
ICS	Field	Virus	Avian/human	α-2,3-/α-2,6-linked sialic-acid-binding IAV	(Watanabe et al. 2015)
ICS	Field	Serum Abs	Avian	H5 and H6 AIV	(Y. L. Cheng, Wang, and Wang 2015)
Multiplex Luminex xMAP	Lab	Serum Abs	Avian	16 HA, 9 NA subtypes	(Germeraad et al. 2019)
Blocking ELISA	Lab	Serum Abs	Avian	H5	(Sączyńska et al. 2021)
Competitive ELISA	Lab	Serum Abs	Avian	H7	(Dong et al. 2019)
ELISA	Lab	Serum Abs	Mammalian	IAV	(Okumura et al. 2019)
Mass spectrometry		Breath	Swine	IAV	(Traxler et al. 2018)
Sound recognition	Field	Sound	Avian	H9N2	(Cuan et al. 2020)

Table 2: Selected developments in IAV-related animal diagnostics since 2015.

H1-subtype diagnostics

The host range of H1 subtypes of IAV includes poultry and wild birds, pigs, cats and dogs, marine mammals and humans. Strains of the H1N1 subtype are endemic in humans, pigs, dogs and birds, and have been responsible for major human pandemics. Among the notable recent developments in testing relating to H1N1 have been two approaches to PCR-based viral identification, which is quicker and more amenable to high-throughput analysis than HI. A multiplex RT-PCR assay has been developed for the simultaneous detection and differentiation of the *HA* genes corresponding to four canine influenza viruses (H1N1/2009, avian-origin canine H3N2, seasonal human-origin H3N2 and equine-origin H3N8) (C. Wang et al. 2017). In this assay, primers specific to the four genes were combined in each reaction, and products were visualised by agarose-gel electrophoresis. The assay was specific for the four canine influenza viruses, including multiple strains of each subtype, with no amplification of the non-canine subtypes H9N2 and H5N1, or of non-influenza viruses. The limits of

detection were 1×10^1 50% tissue-culture infective dose ($TCID_{50}$) for H1N1/2009 and 1×10^0 $TCID_{50}$ for avian-origin canine H3N2, seasonal human-origin H3N2 and H3N8. In 420 clinical specimens, the PCR results agreed completely with those of virus isolation and HI.

An rRT-PCR-based approach has been developed for the detection and subtyping of European enzootic IAV in swine (Bonin et al. 2018). The assay sought to discriminate between avian-origin H1N1, the human-origin 2009 pandemic H1N1, human-like reassortant H1N2, human-like reassortant H3N2 and an antigenic-drift variant of $H1_{hu}N2$. Individual reactions for amplification of the eight *HA* and *NA* genes (avian-origin H1, human-like H1, antigenic drift H1, pandemic H1, H3, N1, pandemic N1 and N2) were refined and validated, along with control reactions for detection of the conserved IAV *M* gene and *Sus scrofa ACTB*. To establish high-throughput diagnostic profiling, the 10 rRT-PCRs were run for each of 118 samples (83 SIV isolates, five SIV mixtures and 30 swine nasal-swab supernatants) in very-low-volume (2 µl) reactions in a PCR machine capable of running 1,536 reactions on a single plate. The *M*-gene rRT-PCR threshold-cycle value was used as an indicator of the level of viral template in each sample, and for clinical samples with a threshold-cycle value <30, the high-throughput analysis enabled full subtyping of >90% of SIV genomes, indicating its potential value for SIV surveillance and epidemiological studies.

Although lab-based diagnostics enable high-precision, high-throughput analyses, they inevitably result in a delay between sampling and diagnosis, and can be associated with high costs when applied to large-scale surveillance. Low-cost, easy-to-use, rapid-turnaround, field-based diagnostics can lead to early identification of infections, enabling actions to be taken to contain potential outbreaks. A point-of-use device for poultry diagnostics has been developed for the electrochemical detection of H1 and H5 HA proteins corresponding to H1N1 and H5N1 AIVs (Veerapandian, Hunter, and Neethirajan 2016). This immunosensor contains two electrodes, each composed of methylene-blue electro-adsorbed graphene-oxide nanostructures, modified with anti-HA monoclonal antibodies, and functionally enhanced by the inclusion of layers of chitosan and protein-A molecules. Immune-complex formation of the sensors with either H1N1 or N5N1 AIVs resulted in differential pulse-voltammetric signals, enabling quantitative discrimination between the viruses over a wide range of concentrations (25–500 pM). This immunosensor had the benefits of simple fabrication and instrumentation, and had an analytical-response time of <1 min.

H3-subtype diagnostics

For the detection of H3-subtype AIV, an anti-H3 mAb has been produced and used for the development of a sandwich ELISA (Luo et al. 2020). In this assay, plates were coated with anti-H3 polyclonal antibody, serum samples were added, and retained H3 AIV was detected by addition of the mAb, followed by an HRP-conjugated secondary antibody. In 180 swab-derived samples, results with the ELISA were coincident with those of viral isolation, HI testing and RT-PCR followed by *HA* and *NA* amplicon sequencing.

The H3-subtype IAVs include H3N2 strains that have avian, swine and canine (and human) hosts, and H3N8 equine and canine strains. In swine (particularly in North America), circulating H3N2 IAV *HA* genes belong to cluster IV, and the subclusters A to F (Grgić, Gallant, and Poljak 2017). Methods for characterization of circulating strains are needed to enable effective and timely vaccination of pigs. A method that has been applied to the detection and differentiation of H3N2 swine IAVs is the polyclonal serum-based proximity ligation assay (polyPLA) (Martin et al. 2017). In this assay, a polyclonal serum (here generated by infection with H3N2 IAV) is split in two, and each half is conjugated with a different oligonucleotide. When the two conjugated polyclonal antibodies are incubated with a suitable antigen (H3N2 IAV), antibody binding brings the oligonucleotides into proximity, enabling their ligation to create a PCR template, which is then used for quantitative determination of the initial quantity of antigen. Application of polyPLA to detection of H3N2 IAV in 81 clinical samples collected from swine at agricultural fairs indicated that specificity of 100% and sensitivity of 77% could be obtained with a ΔC_T cut-off of 7.0. The polyPLA was able to accurately discriminate between H3α and H3β SIVs, with 100% specificity and 85% sensitivity. PolyPLA with polyclonal serum generated from H1N1 SIV was able to identify and discriminate between H1N1 isolates belonging to various antigenic clades. An optimal workflow incorporating polyPLA was suggested to proceed via initial testing for IAV infection, followed by rRT-PCR to determine IAV subtype and then subtype-specific polyPLA for antigenic characterization.

As mentioned in relation to H1 IAV diagnostics, PCR-based assays for the detection of canine H3N2 and H3N8 (C. Wang et al. 2017) and swine H3N2 IAVs (Bonin et al. 2018) have been described. An rRT-PCR assay for the specific identification of H3 AIV subtypes has also been developed (Teng et al. 2015). This assay uses a TaqMan minor-groove-binding probe to generate fluorescence in response to amplification of a conserved region of the *HA* gene of H3 AIV, giving a limit of detection one

thousandth of that of conventional RT-PCR, enabling a much greater detection rate for H3 AIV in samples obtained from oropharyngeal or cloacal swabs from ducks.

Equine influenza results from infection with A(H3N8) viruses that belong to either clade 1 or clade 2 of the Florida sub-lineage. Identification of clade affiliation of isolated EIV is important for epidemiology and for appropriate use of vaccine strains, and it can be achieved by allelic discrimination through a form of multiplex rRT-PCR in which a single amplicon is combined with multiple allele-specific probes. For EIV clade discrimination, Brister et al. assessed the performance of two such assays (Brister et al. 2019). In each assay, a region of the A(H3N8)-virus *HA1* gene containing a clade-specific SNP was amplified by rRT-PCR in the presence of a FAM-labelled clade 1 probe and a VIC-labelled clade 2 probe. Fluorescence signals from the two rRT-PCR assays enabled classification of 341 EIV-positive nasal secretions sampled in the USA from 2012 to 2017 as clade 1 (98.8%) and clade 2 (1.2%), with the clade 2 identifications occurring in horses imported from Europe or vaccinated with a clade 2 modified-live virus.

Rapid antigen detection (RAD) tests are available for diagnosis of equine influenza, but tests with greater sensitivity are required to ensure that the results of field testing can be relied upon to inform the implementation of disease-control measures. In this regard, a RAD test that has been licensed for diagnosis of human seasonal influenza in Japan has been compared against molecular tests and existing equine RAD tests for its performance in the detection of EIV (Yamanaka et al. 2017). The Quick Chaser Auto Flu A, B system uses silver-amplification immunochromatography and densitometric signal measurement to obtain a result for the presence of EIV in a single sample in ≤15 min. In seven horses that were experimentally infected with Florida clade 1 or clade 2 H3N8-subtype EIVs and tested longitudinally for the presence of virus in nasopharyngeal swabs, the average detection period for Quick Chaser Auto Flu A, B was significantly longer than for virus isolation or for conventional RAD tests, and comparable with RT-PCR, rRT-PCR and reverse transcription loop-mediated isothermal amplification (RT-LAMP) tests. On a collection of 550 nasal swabs from pyretic horses testing negative for EIV by RT-PCR, all also tested negative by Quick Chaser Auto Flu A, B.

H5-subtype diagnostics

The H5 subtype of IAVs consists of many antigenic variants, including those that are highly pathogenic in poultry and are responsible for substantial economic loss and depopulation. Although numerous methods are available for subtyping of AIVs, the devastating impact of H5-subtype AIV infections means that there is an ongoing requirement for further assays with the potential to rapidly detect multiple strains of HPAIVs. In addition to the recent development of rRT-PCR assays for specific identification of H5N6 and H5N8 HPAIVs, a multiplex rRT-PCR assay has been shown to have wider specificity for H5 HPAIVs (Le et al. 2020). This multiplex assay contained fluorescence-labelled probes for simultaneous detection of three viral *HA* sequences: one that was conserved across H5 HPAIVs, one that was specific to clade 2.3.4.4 H5 HPAIVs and one that was conserved in clade 2.3.2.1 H5 HPAIVs. Consequently, the assay was able to detect the H5N1-subtype HPAIVs A/environment/Korea/W150/2006 (clade 2.2) and A/duck/Vietnam/NCVD-1648/2012 (clade 2.3.2.1), the H5N6-subtype HPAIV A/Environment/Korea/W541/2016 (clade 2.3.4.4) and the H5N8-subtype HPAIV A/Common Teal/Korea/W555/2017 (clade 2.3.4.4), with no non-specific detection of H1, H3, H7, H9 or H10 IAVs or non-influenza viruses. Notably, the assay did not detect LPAIVs of the H5N2 subtype (A/aquatic bird/Korea/CN2/2009) or the H5N3 subtype (A/aquatic bird/ South Korea/sw007/2015). Another pathotyping/phylotyping rRT-PCR-based method used six separate PCR assays: one *M*-specific reaction for general viral identification, and five reactions to detect potentially zoonotic AIV subtype H5 viruses, enabling distinction between HPAIV and LPAIV, and between clades 2.2.1.2, 2.3.2.1 and 2.3.4.4 (Naguib et al. 2017).

The emergence of new AIV subtypes and reassortment between circulating variants add to the complexity of AIV surveillance in poultry. To facilitate the detection and differentiation of the prevailing AIVs in Taiwan, RT-LAMP coupled with an oligonucleotide microarray has been used for simultaneous subtyping and pathotyping of H5N2, H6N1 and H5 HPAIV variants (with *HA* genes related to an H5N8 isolate) (L. C. Wang, Huang, and Chen 2016). The method required two RT-LAMP reactions to be carried out (using biotinylated primers), one to amplify highly conserved sequences of the *HA* genes corresponding to both H6N1 and H5N2 AIVs, and one to specifically amplify a sequence corresponding to the H5 HPAIV variants. The products of each RT-LAMP reaction were then hybridised to an oligonucleotide-probe microarray and hybridization was visualised via a streptavidin-conjugated alkaline phosphatase colorimetric reaction, the results of which could be read by the naked eye. Different patterns of signals in the seven IAV-specific dots of the microarray corresponded to the presence of H5N2, H6N1 and H5 HPAI viruses, with detection limits of one, one and ten RNA copies,

respectively. This rapid and sensitive assay did not require expensive instrumentation for amplification or for visualization and had great potential for modification to enable detection of emerging AIVs.

Advances have been made in the development of assays for H5 AIVs that may be suitable for use in the field. As described above in relation to H1, one point-of-use device for poultry diagnostics incorporated immune-electrodes responsive to the presence of HA proteins to identify and differentiate between H1N1 and H5N1 AIVs (Veerapandian, Hunter, and Neethirajan 2016). An assay for the specific detection of H5 AIVs has been developed by conjugating monoclonal antibodies against HA of H5 AIV to polydiacetylene vesicles (L. Jiang et al. 2015). Binding of H5 AIV (but not H3 AIV or non-influenza viruses) to the conjugated antibodies resulted in a dramatic blue-to-red colour change within 20 min that could be assessed by eye or by spectrometry, with a limit of detection of 1.35 copies/µl. With a sample set of 93 tracheal swabs collected from wild birds in the field, both virus isolation and rRT-PCR methods identified 14 samples as positive for the presence of H5 AIV, whereas the polydiacetylene biosensor identified 16 positive samples, including two false positives. The ease of use, specificity, sensitivity and detection limit suggest that this biosensor has great potential for rapid, field-based monitoring of H5N1 AIV, facilitating prompt action to control the spread of the virus.

For detection of H5 seroconversion in poultry, a blocking ELISA was developed, in which plates were coated with H5 HA protein, and an anti-H5 mAb was used to block the binding of serum antibodies to the HA antigen (Sączyńska et al. 2021). The assay was validated against 358 serum samples including reference antisera obtained by immunizing SPF chickens with H5N1, H5N2, H5N3 or H5N9 LPAIVs or non-H5 AIVs, experimental antisera from vaccination with recombinant H5 HA protein, and sera from unvaccinated chickens, giving 97.6–100% specificity and 98.0–99.1% sensitivity.

H6-subtype diagnostics

H6 AIVs have low pathogenicity in poultry, but they are also able to cross species barriers into a range of hosts and can provide genes for potentially pathogenic reassortant viruses. As described above in relation to H5 diagnostics, a combination of RT-LAMP and oligonucleotide microarray has enabled simultaneous subtyping of H6N1 and H5 AIVs (L. C. Wang, Huang, and Chen 2016).

An immunosensor that has been validated for the detection of H6N1 AIV used the phenomenon of surface plasmon resonance to measure binding of virus to a monoclonal antibody that had been conjugated to a gold-coated exposed surface of an optical fibre (X. Zhao et al. 2016). The detection

limit of the immunosensor for A/chicken/Taiwan/2838V/00 (H6N1) was 5.14×10^5 EID_{50}/0.1 ml, with a test time of 10 min, which compared with 2.10×10^6 EID_{50}/0.1 ml and 3 h for antigen-capture ELISA and 2.60×10^4 EID_{50}/0.1 ml and 2.5 h for RT-PCR. The optical-fibre immunosensor was a low-cost, label-free device requiring only 50 µl of sample. Furthermore, with the appropriate washing steps, each sensor could be reused multiple times.

Although HI is the gold-standard test for seroconversion resulting from IAV infection, it is a time-consuming assay that must be carried out in a laboratory. An immunochromatographic-strip test was therefore developed to enable the rapid, pen-side assessment of serum antibodies to H6N1 AIV (Y. L. Cheng, Wang, and Wang 2015). In this lateral-flow assay, A/chicken/Taiwan/2838V/2000 (H6N1) virus was bound to the test line of a nitrocellulose membrane, and the formation of complexes between the immobilised virus and anti-AIV serum antibodies was detected by the inclusion in the strip of colloidal-gold-conjugated antibodies against chicken IgG. Seroprevalence of antibodies against H5 and H6 AIVs in 326 field samples of chicken serum was determined by HI, and compared with the results of the same samples with the immunochromatographic-strip test. Relative to the HI results, the immunochromatographic-strip test had sensitivity of 95.2% and specificity of 94.3%, demonstrating that it could detect antibodies to both H5 and H6 AIVs. This assay was simple and rapid, requiring neither equipment nor skilled operators, giving it great potential for field diagnostics.

H7-subtype diagnostics

H7 LPAIVs can give rise to HPAIVs via spontaneous mutations that result in the insertion of basic amino acids at the cleavage site of the HA protein. H7 AIV infections are therefore notifiable to the OIE, and pathotyping by characterization of the HA cleavage site is very important for management of confirmed H7 AIV infections. Pathotyping by determination of the IVPI in experimentally inoculated chickens requires appropriate animal-experimentation facilities, and pathotyping by nucleotide sequencing requires expensive laboratory equipment. To overcome these limitations, an rRT-PCR-based method for H7 IAV pathotyping has been demonstrated (Graaf, Beer, and Harder 2017). By amplification of a short fragment of the *HA* gene spanning the sequence encoding the endoproteolytic cleavage site in the presence of nuclease-activated, fluorescence-labelled cleavage-site-specific probes, the assay could distinguish the presence of H7 LPAIV (of the H7N1, H7N2, H7N3, H7N4, H7N7 and H7N9 subtypes) in 48 of 56 samples, and H7 HPAIV (of the H7N1 and H7N7 subtypes) in 15 of 19 samples, with additional discrimination between two H7 HPAIV lineages. Because detection was highly

lineage-specific, it was suggested that this assay would be used following an initial sequence analysis of any newly identified infection, to enable the design of specific probes.

In countries that are at risk of the introduction of H7N9 AIV, active surveillance has been performed, but its value has been limited by the length of time required to send samples for processing and to obtain results. Rapid-turnaround assays are required, and in particular, assays that can be conducted in the field. Insulated isothermal RT-PCR (iiRT-PCR) systems are portable, battery-powered devices that use freeze-dried, thermostable reagents to carry out convection-driven PCRs in <1 h. Coupled with a portable nucleic acid extraction system, an iiRT-PCR assay has been developed and shown to have 100% specificity and sensitivity for the detection of both H7 *HA* and N9 *NA* sequences in extracted viral reference RNA, with 100% specificity and 98% sensitivity for the detection of H7 AIVs in extracts of oropharyngeal samples from chickens and ducks (Inui et al. 2019). The system has been trialled and shown to be suitable for use in the field.

Another nucleic-acid amplification procedure that has been adapted for H7N9 AIV diagnostics is recombinase polymerase amplification (RPA), an extremely versatile and rapid isothermal assay in which recombinase enzymes facilitate binding of oligonucleotide primers to target DNA sequences, thereby enabling DNA amplification by polymerase action. By establishing RPA reactions for the H7 *HA* and N9 *NA* genes, with each reaction containing an unlabelled forward primer, a biotin-labelled reverse primer and a FAM-labelled probe, specific RPA amplicons could be detected by applying the reactions to a lateral-flow device (S. Ma et al. 2018). Carbon-conjugated neutravidin in the test strip bound to the biotin, and when the complexes reached the test line, they were captured by anti-FAM antibodies, so that only an appropriate amplicon containing both biotin and FAM would concentrate carbon particles at the test line to give a visible signal. The RPA reactions took 20 min at 39°C, with a further minute for the lateral-flow assay. The assay could detect 32 fg of cDNA derived from H7N9 viral RNA, and was specific for H7N9, with no detection of the *HA* or *NA* of H1N1, H3N2, H5N6 or H9N2 subtypes.

ELISAs are available for both antigenic and serological diagnosis of infection with H7-subtype AIVs. A sandwich ELISA used a pair of anti-H7 mAbs, and had detection limits of 0.5–2.0 HAU/50 µl for H7N7 and H7N9 live viruses (Lingling Chen et al. 2019). A competitive ELISA that used an anti-HA mAb to detect serum antibodies to H7 AIV was validated with 1,294 field samples from chickens, ducks, geese, quails and pigeons, and had specificity of 93.2% and sensitivity of 96.2%, with a coincidence rate of 97.5% in comparison with HI (Dong et al. 2019).

Immunological point-of-care tests have been developed for the detection of H7N9 AIV. For one test, two mouse monoclonal antibodies (mAbs) were produced to H7N9 recombinant HA1 protein (Yeo et al. 2017). One mAb (2F4) was conjugated to the fluorescent reagent Europium and used to impregnate the conjugate pad of an immunochromatographic strip, and the other mAb (6D7) formed the test line of the strip. When a sample containing H7 AIV was applied to the strip, the virus bound to the Europium-conjugated 2F4, then migrated to the test line and bound to 6D7, giving a fluorescent signal at the test line that could be read by an LED-based portable strip reader in 15 min. The optimised assay had a limit of detection of 40 HAU/ml for two H7 subtypes (H7N1 and H7N7), and did not give a test signal with H1N1 or H5N3 AIVs. Although widespread use of this test could be restricted by the cost of the LED reader (~20,000 USD), there is potential to adapt the assay for use with a smartphone-based imaging system. Another assay has been specifically designed to overcome the problems of low signal intensity and the unsuitability of imaging devices to field deployment that are associated with the use of immunochromatographic strips. In this assay, highly fluorescent (CdSe)ZnS quantum-dot nanobeads were conjugated with mAb to H7N9 AIV, and used to impregnate the conjugate pad of an immunochromatographic strip (M. Xiao et al. 2019). H7N9 virus in a sample added to the strip formed a complex with the mAb–nanobeads, and on migration through the strip, formed a further complex with mAb immobilised at the test line. To facilitate on-site analysis, a compact fluorescence imaging unit was designed and 3D-printed. The unit functioned as a darkroom that contained battery-powered LEDs for fluorescence excitation, enabling read-out by eye (and image capture by smartphone camera) in 15 min. With a commercial strip reader, the limit of detection for H7N9 virus was 0.0268 HAU, and in the 3D-printed reader an unambiguous positive signal was produced with as little as 0.0312 HAU. The assay did not detect H1, H5 or H9 AIVs or non-influenza viruses, and in 50 H7N9 samples from different chicken organs it had a total coincidence rate of 98% with rRT-PCR.

H9-subtype diagnostics

H9N2 LPAIVs on their own are responsible for mild-to-moderate disease in poultry, but co-infections with other viruses and bacteria can result in around 70% morbidity and 30% mortality. In addition, some H9N2 viruses recognise human-type α-2,6-linked sialic acid receptors, and avian-to-human transmission of Eurasian-lineage H9N2 AIV has been observed. To improve monitoring of these viruses, a one-step rRT-PCR assay has been developed for detection of two major Eurasian H9N2 lineages of AIV (Saito et al. 2019). The assay incorporated a minor-groove-binding probe, and had a

detection limit with *in vitro*-transcribed RNA of around three copies per reaction, with no cross-reactivity with other IAV subtypes, IVB or other viral respiratory pathogens.

As described above in relation to H7N9 diagnostics, RPA can be combined with lateral-flow assays to enable the rapid, field-based detection of AIVs (S. Ma et al. 2018). This combination has also been applied to assessment of H9N2, and specifically to the amplification of a sequence of the H9 *HA* gene, using a forward primer labelled with fluorescein isothiocyanate and a reverse primer labelled with biotin (Zeng Wang et al. 2019). The amplification reaction took 20 min, and the lateral-flow assay could detect 0.15 pg of H9N2 AIV RNA. With 120 cloacal samples from chickens with respiratory syndromes, the coincidence rate of the RPA assay and conventional RT-PCR was 95.8%.

A sandwich ELISA using a pair of mAbs has been developed for detection of H9N2 AIV (Ming et al. 2019). The assay had a detection limit of $10^{-2.3}$ $TCID_{50}$, with 98.9% sensitivity and 98.1% specificity relative to detection based on virus isolation.

Smartphones have great potential to facilitate field-based diagnostic imaging. For the detection of H9N2 AIV, smartphone-based imaging has been coupled to an immunochromatographic-strip test. This assay used an anti-NP antibody to form a complex between the virus and a fluorescent bioconjugate, and also to capture that complex at the test line of the strip (Yeo et al. 2016). Fluorescence signals from the test and control lines were generated in a compact, lightweight, 3D-printed module that contained an LED and optical filters of appropriate wavelengths. The LED was powered by connection to a smartphone, which was also used as an imaging system, to visually record the result, to calculate the ratio of the test-line and control-line signals, to make a binary decision on positive-versus-negative detection, to log the results along with the date of testing and the location of the testing site, and finally to submit the results to a central database. The detection limit of the assay was 7.5 PFU/ml, with specificity of 100% and sensitivity of 94.4% for oropharyngeal samples and 95.2% for cloacal samples, relative to rRT-PCR results.

H10-subtype diagnostics

The AIV subtypes H10N4, H10N5, H10N7 and H10N8 consist mostly of low-pathogenicity viruses, but high-pathogenicity H10 AIVs have been identified, and H10 AIVs are also capable of causing infections in mammals, including pigs and humans. To facilitate monitoring of H10 AIVs, RT-LAMP has been used, with primers specific to conserved sequences of the H10 *HA* gene (Luo et al. 2015). The assay took 40

min at 63°C to give a result that could be observed directly as a colour change from orange to green, or that could be measured in real time by assessment of turbidity. The limit of detection of the assay was 10 copies/µl of *in vitro*-transcribed RNA, and it demonstrated specific amplification of H10-subtype AIVs, with no cross-reactivity with other AIV subtypes or with non-influenza avian pathogens. The assay can provide a rapid, simple, low-cost test with potential for use in the field.

Diagnostic tests for multiple IAV subtypes

Reassortment and mutation increase the numbers of circulating IAV subtypes, along with the potential for the occurrence of highly pathogenic and/or zoonotic strains, and the need for diagnostic assays that are capable of identifying multiple relevant subtypes. For example, at least eight AIV subtypes (H1, H2, H3, H5, H6, H7, H9 and H10) have been found to infect humans, and an assay has therefore been developed for the simultaneous detection of all of these eight subtypes (M. Li et al. 2018). The basis of the assay was the multiplex RT-PCR amplification of a specific *HA* region for each subtype, and detection was achieved by capillary electrophoretic separation of amplified products and spectrophotometry to identify the incorporated fluorescent labels, using the GeXP system. The assay could detect as few as 100 copies of RNA templates, and was not affected by the presence of RNA from multiple viral subtypes. With specimens from domestic poultry and wild birds, the assay specificity was equivalent to that of conventional RT-PCR.

NA subtyping of IAVs has traditionally been performed by neuraminidase-inhibition assays, but that approach is not ideal, because of the time and labour it requires, as well as issues of diversity of reference antisera, cross-reactivity, subjectivity and accuracy. An rRT-PCR-based method has made it possible to detect nine *NA* subtypes, with a multiplexed setup involving three reactions per sample, each with three primer–probe–fluorescent-label combinations (Z. Sun et al. 2017). The assay had a detection limit of <100 EID_{50} or <100 copies of cDNA per reaction, showed no subtype cross-reactivity, and had comparable sensitivity to sequencing of isolated virus when applied to tracheal and cloacal swabs of experimentally infected chickens.

The Riems Influenza A Typing Array (RITA) combined 32 individual TaqMan rRT-PCRs: one each for detection of H4, H6, H8, H9, H11, H12, H13 and H16; two each for H1, H2, H3, H5 and H10; three for H7 detection; one each for subtyping of N1, N2, N4, N5, N6, N7, N8 and N9; two assays for N3; along with amplification of an *M* gene sequence for pan-influenza IAV detection (Hoffmann et al. 2016). The

assay was validated with isolates representing 45 different IAV subtypes, with diagnostic samples obtained from wild birds and swine.

Assays are sometimes developed to target specific combinations of IAV subtypes. For example, an assay developed to facilitate the monitoring of migratory birds in Taiwan used multiplex RT-RPA to detect the conserved *NP* gene and the *HA* genes of clade 2.3.4.4 H5, H6 and H7 subtypes (Tsai et al. 2020). By coupling the RT-RPA to capillary electrophoresis for high-throughput analysis of the reaction products, a detection limit of one gene copy could be achieved in a total assay time of 6 h. With 60 field samples collected from migratory wild ducks or endemic waterfowl, the multiplex RT-RPA assay identified the presence of AIV in 50 samples by positivity of *NP* and at least one *HA* product. Furthermore, in 44 of the samples, the assay identified co-infection, with 42 of the samples being positive for H5, H6 and H7 products. Another multiplex assay for specific IAV subtypes has targeted those that are relevant to commercial pig farming in Denmark (Goecke et al. 2018). In this high-throughput method, 18 rRT-PCRs were carried out for each sample, with five reactions targeting specific *HA* gene sequences corresponding to avian-origin H1N1, human-like H1N2, A(H1N1)pdm09, H3N2 SIV and seasonal human H3 IAV, six reactions targeting N1 and N2 *NA* sequences (two broadly reacting assays for each subtype, one reaction specific for A(H1N1)pdm09-lineage N1 and one for seasonal human H3N2), one reaction for a conserved *M* sequence, and six reactions that were specific for the internal genes of A(H1N1)pdm09 (*PB1*, *PB2*, *PA*, *NP*, *M* and *NS*). Following an initial pre-amplification step, the reactions were carried out with the high-throughput BioMark platform, and showed high sensitivity and specificity, with no cross-reaction, and with the detection of co-infections.

For some purposes, the aim is to identify infection with any IAV, rather than with a particular subtype. In relation to AIV, RT-LAMP has been used for amplification of a conserved region of the *M* gene, enabling the detection of H1–H16 subtypes following a 30-min reaction (SHI et al. 2019). Inclusion of fluorescein enabled visual identification of amplification, and the reactions could also be monitored by measuring turbidity, which gave them a detection limit of 0.1 PFU per reaction. The RT-LAMP detection rate among 335 clinical samples (throat swabs, tracheal aspirates and visceral organs) was the same as with rRT-PCR, and higher than that with conventional RT-PCR (14, 14 and 12 positive results, respectively). A very different approach has been taken to detection of the NP protein of swine IAV (D. Su et al. 2019). The assay that was used was based on the giant magnetoresistance effect, in which the electrical resistance associated with a structure composed of alternating ferromagnetic and non-magnetic conductive layers depends on whether the magnetization of the ferromagnetic layers is in a parallel or antiparallel alignment. This structure was coated with an antibody to IAV NP, and the

test sample was incubated with magnetic nanoparticles coated with another anti-IAV antibody, then added to the magnetoresistance sensor. Binding of IAV–nanoparticle complexes to the sensor could be detected by applying a magnetic field and measuring the change in magnetoresistance. This assay enabled the technically simple, wash-free analysis of swine nasal-swab samples, with a limit of detection of 250 $TCID_{50}$/ml for swine IAV and a procedure time of 4 min in a handheld, portable device capable of wireless communication. Notably, ELISAs are also available targeting conserved viral proteins or serum antibodies to those proteins, for antigenic or serological detection of multiple IAV subtypes.

Several assays exist for the simultaneous detection of IAV and other viral and/or bacterial pathogens. Although some of these assays target a single, conserved IAV sequence, others incorporate some form of IAV subtyping. One example of a test for important avian pathogens involved the RT-PCR amplification of sequences for the differentiation of the H5, H7 and H9 subtypes of AIV, as well as NDV and infectious bronchitis virus (Q. Xiao et al. 2019). Biotin-labelled amplified products were hybridised to specific oligonucleotide probes in a microarray, and hybridization was detected by the addition of streptavidin conjugated to horseradish peroxidase, giving a visible signal that could be assessed by eye. There was no cross-reaction with other subtypes of AIV or with IBDV, and the detection limits for H5, H7 and H9 AIV and NDV were 0.1 EID_{50} per reaction (1 EID_{50} per reaction for infectious bronchitis virus).

Multi-strain serology

Tests for the identification of seroconversion resulting from IAV infection include both non-specific and multiplex assays. For AIV, a multiplex Luminex xMAP assay has been developed for the simultaneous subtyping of antibodies to any of 16 HA and 9 NA subtypes (Germeraad et al. 2019). Recombinant HA and NA proteins were coupled to colour-coded magnetic beads, and following incubation with poultry sera, complexes of antigen, bead and antibody were further bound to fluorescence-labelled anti-chicken-IgY antibodies. The association of a specific colour with each antigen then enabled its fluorescence signal to be measured, indicating the presence of antibodies to any AIV subtype in the sample. This assay gave results that were consistent with those of HI and ELISA in 97.8% of reference sera and 90.8% of field sera. There was no cross-reaction with non-influenza avian respiratory viruses, and the detection limit was lower than that of HI, and was achieved with a much lower (2 µL) sample volume. The multiplex assay has the further advantage that it is able to identify antibody profiles that indicate infection with multiple viral subtypes in a single animal.

An ELISA for serological detection of infection by multiple subtypes of IAV was developed by coating plates with H1–H15 HA proteins (Okumura et al. 2019). Reference antisera demonstrated specific reactions with these antigens, and the assay enabled serological subtyping in samples from wild boars and from raccoons, suggesting the potential for the use of this ELISA for sero-surveillance of wild mammals.

Further advances in IAV diagnostics

The zoonotic and pandemic potential of IAV is of great concern, and has been addressed by the creation of a number of assays that identify HA binding specificity for sialic-acid-terminated glycans. AIVs are generally characterised by binding to α-2,3-linked sialic acid, whereas a switch to binding α-2,6-linked sialic acid facilitates human infection. Notably, both types of linkage are present on respiratory epithelia in swine. A device that has been evaluated for the discrimination of binding preferences to these glycan types is the dual-channel field-effect-transistor biosensor (Hideshima et al. 2019). The biosensor was generated by the binding of sialic acid-α-2,6-galactose to one gate and sialic acid-α-2,3-galactose to the second gate of the transistor. IAV samples in human nasal mucus (treated with L-cysteine ethyl ester to reduce viscosity) were added to the glycan-immobilised biosensor gates for 10 min, the sensor was washed, and the threshold voltage shift was calculated for each gate, as a measure of virus binding. The human H1N1 IAV was specifically detected by the sialic acid-α-2,6-galactose gate, whereas H5N1 AIV was detected by the sialic acid-α-2,3-galactose gate, with a detection limit of $10^{0.5}$ $TCID_{50}$/ml. When the binding of NDV was assessed as a model for a pandemic IAV (NDV recognises both α-2,6-linked and α-2,3-linked sialic acids), it gave a signal at both gates of the sensor. The system was evaluated for potential use in the field, and the modified transistors were found to maintain their effectiveness even with extended storage, and to integrate well with a handheld reader that connected via Bluetooth to a smartphone.

A variation on the immunochromatographic-strip method has also been used for identification of IAV receptor-binding specificity (Watanabe et al. 2015). In this assay, a biotinylated broad-specificity (group 1) anti-HA antibody enabled capture of IAV at the test lines of the strips. Inclusion of blue-latex-conjugated α-2,3-sialylglycopolymer or red-latex-conjugated α-2,6-sialylglycopolymer (on separate strips) meant that AIV could be distinguished by a blue test line on the α-2,3 strip, whereas human IAV gave a red test line on the α-2,6 strip. The presence of these coloured test lines on both strips indicated that the sample contained interphyletic virus with affinity for both types of receptor. The assay

showed great potential for use in the field, with no deterioration of the strips on long-term storage, no requirement for specialised equipment, and a test time of only 30 min.

Direct detection of viral proteins or nucleic acids or serum antibodies is not the only way to test for IAV infection. For example, a pilot study has been carried out to investigate the potential association of IAV with the presence of volatile organic compounds in the breath of swine (Traxler et al. 2018). These compounds were analysed by gas-chromatography mass spectrometry, and six (acetaldehyde, propanal, n-propyl acetate, methyl methacrylate, styrene and 1,1-dipropoxypropane) were found to be related to influenza disease progression, with the potential to enable early detection of IAV infection. However, although the analysis of volatile organic compounds in swine breath is non-invasive, the breath-capture method used in the pilot study required harnessing of individual pigs in a sling and fitting with a respiratory mask, and further improvements are clearly necessary to enable the continuous, large-scale screening that would maximise the potential of this approach.

In the search for non-invasive diagnostic methods, it has been discovered that analysis of the sounds made by chickens might enable identification of AIV infection (Cuan et al. 2020). A sound-recognition method called the chicken sound convolutional neural network has been developed and combined with the separation of chicken sounds from environmental sounds (to reduce redundant data processing) and with mapping of specific features of the chicken sounds. Different data-processing models were assessed, and the chicken sound convolutional neural network with input via spectrogram achieved accuracies of 93%, 95% and 97% on days 2, 4 and 6 post injection for discrimination of uninfected chickens from chickens infected with H9N2 AIV. With input via feature mapping, the recognition accuracies were 90%, 94% and 96% on days 2, 4 and 6, with the additional benefit that feature mapping had the fastest training speed and smallest data size of the pre-processing techniques. Sound-based AIV diagnostics could provide continuous monitoring of flocks without the need for any physical testing of individual birds, thereby reducing the risk of human infection and potentially greatly reducing the costs associated with surveillance.

Not all methods that are assessed for IAV diagnostics result in successful applications. Body temperature has been found to be ineffective for the screening of swine at agricultural fairs, where early detection would enable prevention of transmission among pigs and between pigs and humans (Bowman et al. 2016). Surface body temperatures were measured with infrared thermometers in 1,092 pigs and compared with the results of nasal swabs. Similarly, rectal temperatures were measured in 1,948 pigs and compared with results from snout wipes. In both cases there was no

significant difference in mean temperature between uninfected pigs and those infected with swine IAV.

Diagnostic sampling and sample storage

Lab-based diagnostics rely upon the stable transportation of biological samples, to maintain the integrity of the testing analytes. Developments that improve sample stability have potential benefits for many diagnostic systems, particularly if they can also increase transportation biosafety. Flinders Technology Associates filter paper cards are formulated to denature proteins, lyse cells and bind nucleic acids, and they have been assessed as sampling media for numerous pathogens, including H7N1 LPAIV and H5N1 HPAIV (Jóźwiak et al. 2016). Following application of viral samples to the cards, complete inactivation of H7N1 LPAIV was observed within 1 h storage at room temperature, whereas the H5N1 HPAIV was inactivated by the second timepoint of 24 h. Viral RNA could be recovered from the cards following room temperature storage of at least 150 days, and was suitable for use in rRT-PCR detection and subtyping assays.

For the purposes of diagnostic analysis, sampling methodology is a relevant consideration. A long-term, single-site surveillance study in which 26,586 samples from wild mallards were screened by rRT-PCR for the presence of AIV demonstrated that cloacal/faecal samples were associated with consistently higher detection rates and lower PCR C_T values than oropharyngeal samples (Latorre-Margalef, Avril, et al. 2016). Among 4,354 sample pairs with both oropharyngeal and faecal/cloacal samples, 80.5% gave negative rRT-PCR results in both samples, 3.5% were positive in both, 13.0% were positive in the faecal/cloacal sample but negative in the oropharyngeal sample, and 2.9% were negative in the faecal/cloacal sample but positive in the oropharyngeal sample. The success rate of virus isolation in embryonated chicken eggs was negatively correlated with rRT-PCR threshold cycle and with age, as samples from adult birds resulted in lower success rates than comparable samples from juveniles. The isolation success rates were also greater in faecal samples than in comparable cloacal samples. These results suggested that the ideal sampling regimen in diagnostic terms would involve both cloacal and oropharyngeal samples for each bird, and that such an approach would be necessary to maximise the probability of HPAIV detection (because of respiratory tract tropism), but if sampling was restricted to a single sample per bird and the aim was the detection of LPAIV, then cloacal samples would be preferable. The results also indicated the importance of the collection of metadata such as host species and age, and sample type, to ensure analytical consistency between surveys.

Detection of subclinical infections is a challenge for approaches that use sampling of individual animals. In turkey flocks affected by LPAIV infection, viral RNA has been shown to be widely distributed in the barn environment, including in biofilms associated with the drinker apparatus (Muñoz-Aguayo et al. 2019). Viral RNA could be detected in these biofilms by rRT-PCR and by virus isolation, and correlated with results from oropharyngeal samples. At an HPAI-positive site, virus was detected in biofilm swabs 1–2 days prior to its appearance in oropharyngeal samples.

PCR primer validation, internal controls and viral RNA enrichment

IAVs are constantly evolving, and established diagnostic tests need to be assessed regularly to ensure that they still perform optimally in recognition of the currently circulating viral strains. A number of existing RT-PCR methods for IAV diagnosis are based on amplification of the conserved *M* gene, and four such protocols that are routinely used in AIV reference laboratories, along with a USDA-licensed commercial kit, have been evaluated in silico and *in vitro* to determine the level of identity between their primers and probes and the target regions in known *M* gene sequences (Laconi et al. 2020). These assays were found to have differing levels of diagnostic sensitivity, and lower sensitivity was associated with viral sequence variability within primer binding sites. The more recently developed assays showed the best analytical and diagnostic performances, suggesting the need to update the primer and probe sequences for the older assays. In a study with a similar aim, the *M* gene PCR primers for 12 assays were compared with 9,103 complete *M* gene sequences (J. W. Kim et al. 2019). None of the primers was found to have 100% identity with all of the gene sequences; the 100% identity rate for individual forward primers ranged from 0 to 93.2%, and for reverse primers it ranged from 0.4% to 85.3%. A new set of degenerate forward and reverse primers was designed with 100% identity rates of 94.4% and 96.2%, respectively. The primers were incorporated in a SYBR Green-based rRT-PCR assay, and sensitivity was further increased by the use of immunomagnetic beads to concentrate the viral ribonucleoprotein prior to RNA extraction and PCR.

Sequencing of IAV RNA enables subtyping and identification of new mutations and reassortants. Because PCR amplification of limited amounts of RNA template may introduce sequence errors, and to enable enrichment of any IAV RNA without prior knowledge of the subtype, a set of universal oligonucleotide probes has been designed for the hybridization capture of any IAV, IBV or ICV sequences (Yongli Xiao et al. 2018). The set of 46,953 120-nt biotin-labelled RNA probes was based on

all the available influenza viral sequences, and its use with wild-bird cloacal-swab samples enabled detection of mixed infections with different AIV subtypes.

To ensure that RT-PCR test results can be accurately compared, both within and between labs, appropriate internal controls should be used. An exogenous, 'armoured' internal control consisting of a 65-nt sequence of the gene encoding enhanced GFP has been produced for use in IAV RT-PCR assays (Andreychuk et al. 2019). The 'armour' was branched polyethylenimine, which conferred resistance to nucleases and physical stability, enabling the control to be added directly to pathological material suspensions, thereby facilitating assessment of the efficiency of subsequent extraction of viral RNA. Amplification of the control RNA with specific primers resulted in consistent threshold-cycle values in samples from different species of wild birds. Addition of PCR inhibitors resulted in increased threshold-cycle values for the internal control.

Ongoing research

Diagnostic virology researchers at the Friedrich-Loffler-Institut are working to improve diagnostic procedures within the framework of the national and OIE/FAO reference laboratory. At the National Veterinary Research Institute in Vom, Nigeria, study is ongoing into rapid laboratory diagnosis of HPAI via PCR and virus isolation. Research at the Norwegian Veterinary Institute, meanwhile, continues as part of TELE-Vir, an initiative of the One Health European Joint Programme. A critical aim of this project is to develop field-side diagnostic tools based on 3rd-generation sequencing technology for efficient identification and characterisation of diseases like influenza. Data can then be collected and analysed within the user-friendly web-based Insaflu portal, easing international collaboration efforts.

At the UK Animal and Plant Health Agency, meanwhile, researchers are working to enhance the influenza diagnostic profile by expanding molecular detection and subtyping methods, validating novel molecular assay formats, and optimizing sample submission pipelines. At the US Southeast Poultry Research Laboratory, efforts are underway to improve real-time PCR diagnostics for AIV, evaluating existing technologies and developing reagents for subtype-specific tests. Next-generation sequencing is also under study, with researchers working to increase its sensitivity and efficiency and improve data analysis.

In France, the Ploufragan-Plouzané-Niort Laboratory of ANSES has served as the nation's SIV reference laboratory for over ten years, running a network of veterinary labs approved for molecular diagnosis,

providing reference reagents and technical support/expertise, and performing inter-laboratory tests to ensure continuing aptitude within the network. Similarly, at the US National Poultry Research Center, established diagnostic tests for AIV are monitored and updated to reflect changing viral transmission patterns. Novel test formats are also evaluated using a combination of *in vitro* and *in vivo* methods.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of animal influenza diagnostics should be considered priorities for future research:

Application of SARS-CoV-2-related advances in diagnostics to IAV
Identification of novel reassortants and mutations with pathological potential
Continued improvement of diagnostics for subtyping, serology and whole-genome sequencing
Use of portable 3rd-generation sequencing technology to enable point-of-incidence analysis
Standardization of methods across labs
Rapid characterization and pathotyping of variants circulating in the field (high throughput point-of-use technology)
Fast typing of AI virus using molecular tools without virus isolation, applicable for noninvasive samples from wild birds.

Vaccines

Vaccines are an important component of comprehensive influenza control strategies that may be used in both enzootic and epizootic situations. However, current vaccines suffer several shortfalls across the various target species: breadth and duration of protection are insufficient, the vaccines can account poorly for the currently-circulating strains, and obstacles exist to rapid production and dissemination in the face of an outbreak, particularly in the case of egg-produced AIV vaccines.

Improved vaccination strategies are urgently needed to prevent unnecessary mass culling of livestock due to lack of sterile immunity, to reduce the economic and social burden of influenza infection, and to decrease the risk of zoonotic infection of humans. The success of these strategies will be underpinned both by studies of immunity to natural influenza virus infection in host species, as well as by careful investigation of the immune responses induced by various novel vaccine candidates to establish correlates and/or underlying mechanisms of durable protection and cross-protection. The application of computer modelling to this task holds promise of rapid advances in the coming years, if used and tested well. However, significant regulatory and practical challenges remain before we will be able to realise the ultimate goal of easily administered, effective vaccines for all affected species.

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps in animal influenza vaccines in 2014/15:

- *novel technologies that reduce the time taken to produce a vaccine*
- *development of vaccines/adjuvants to induce broad/universal clinical cross-protection*
- *strategies to increase mucosal immune responses after inactivated vaccines*
- *role of unregulated cytokine expression in production of vaccine-associated enhanced respiratory disease of swine*
- *multi-species vaccine platforms*
- *vaccines with accompanying DIVA tests*
- *needle-free mass-delivery systems for vaccines*
- *immune correlates of vaccine-induced protection*

- *strategies to improve vaccine responses* in ovo *or in young animals, especially in the presence of maternal antibody*
- *strategies to increase speed of protection from vaccines*
- *vaccine performance under field conditions*

Literature review

Avian Influenza Vaccines

Route of delivery

Conventional inactivated AI vaccines require cold-chain storage and injection by well-trained personnel: this dual reliance has the potential to lead to reduced vaccine efficacy, as well as being time consuming and leading to the generation of huge quantities of biohazardous sharps waste globally.

Powder vaccines – permitting long term storage capacity with no need for a cold chain – are immunogenically and physically stable at up to 30°C for 3 months (Murugappan et al. 2013). Building on their previous work showing partial protection of chickens by passive inhalation of freeze-dried inactivated H5N1 (Peeters et al. 2014), Tomar et al. compared the passive inhalation of inactivated freeze-dried reverse-genetics-derived H5N1/H1N1 virus alone to the inhalation of the same inactivated virus adjuvanted with *Lactococcus lactis*-derived bacteria-like particles (BLP) or adjuvanted with the commercially available inulin-based adjuvant Advax^{TM} (Tomar et al. 2018). The researchers found that the addition of either adjuvant did not adversely affect the vaccine's physical properties and both induced significantly higher titres of specific circulating antibody in immunized hens as well as mucosal IgY (BLP) and IgA (Advax) in the lung compared to the non-adjuvanted, inactivated virus: the best results were obtained when BLP were used in the booster dose. When chickens that had received two doses of the vaccines two weeks apart were challenged two weeks later, all birds were completely protected from HPAI and no viral shedding was detected, regardless of whether the vaccine included adjuvant or not. The researchers proposed that this difference from the previous study was likely related to the reduced particle size of the powder, which aimed to improve vaccine delivery into the lung, and the increased concentration of the powder in the immunisation chamber (Tomar et al. 2018). The inclusion of adjuvant might still be useful to either reduce the amount of inactivated virus needs for an effective does (important in outbreak situations), to speed induction of protective immunity, and/or to increase the length of effective immune memory following

vaccination. These points require assessment in further studies, which are highly warranted given these promising data.

Coarse spraying is another method of vaccine delivery suitable for mass application. Ma et al. showed that an attenuated NDV (LaSota strain) vectored vaccine expressing the ectodomain of HA from H5N2 could clinically protect 90% of chickens from H5NX challenge, and almost completely block viral shedding, after a single application (Jingjiao Ma et al. 2017). As for the Tomar et al. study above, further investigations are needed but this promising method should be more widely explored for AIV vaccine delivery. Future studies should also assess the possible impact of existing immunity to NDV (either due to natural infection or vaccination) upon responses to attenuated NDV-vectored vaccines in commercial chicken flocks in the field.

Field studies

Given the numerous differences between laboratory and field conditions, it was reassuring that a recent study testing two different inactivated H9N2 vaccines showed comparable immune responses and reduced viral shedding from chicks immunized between 1 and 7 days of age and challenged at 28 days of age under either laboratory or field conditions (Talat et al. 2020). Importantly, all chicks were from vaccinated hens and therefore exposed to MDA, but effective vaccination responses were mounted following immunization at 7 days old, though less so if chicks were vaccinated at 1 day old (Talat et al. 2020).

A single study reported a full field trial of an experimental inactivated fowl cholera and AI H9N2 vaccine in poultry, followed by laboratory transfer for challenge (Salama, Abdelhady, and Atia 2019)while another study in ducks on smallholdings demonstrated the high immunogenicity of the current H5N1 vaccine in use in Vietnam's HPAI control program (Huynh et al. 2019).

Tarigan *et al.* measured the duration and levels of immunity stimulated by vaccination with seven different commercially available inactivated H5N1 vaccines in 16 small-to-medium sized commercial laying flocks in Indonesia (Tarigan et al. 2018). They found marked differences in strength and duration of immunity, as well as significant variations between farms whose cause was unknown (Tarigan et al. 2018). An important finding was that few farms followed the manufacturer's recommended dosing schedule; moreover, complete vaccination failure in two farms was linked to errors in vaccine administration. Just two of the 16 flocks demonstrated antibody titres associated with protection at the end of the sampling period, which the researchers linked to repeated vaccinations: they suggested

that a dosing schedule incorporating five immunizations would give the greatest chance of long-lasting immunity at the flock level which would be effective in preventing spread of HPAI (Tarigan et al. 2018). Similarly, Sitaras *et al*. explored the impact of sub-optimal field-like vaccination upon cross protection from challenge with antigenically-distant viruses (Sitaras et al. 2016). The authors concluded that, for the viruses tested, even a low level of immunity had some cross-protective effect, and suggested that better cross-protection could be induced simply by improving the performance and administration of current vaccines. However, this study also highlights the often-overlooked (or at least unquantified) human factors involved in vaccine efficacy.

In a rare but much-needed approach, Chen et al. recently extended their laboratory-based vaccination study (P. Chen et al. 2019) to the field, immunising 500 farmed ducks three times with a recombinant duck enteritis virus expressing HA from H5N1 HPAI and demonstrating efficient induction of antibody responses across the cohort (P. Chen et al. 2019). The researchers then transferred groups of ducks to the laboratory for challenge experiments and observed sterile protection from homologous virus and complete clinical protection with at least 80% reduction in frequency of shedding ducks after heterologous H5N6 and H5N8 AI (J. Wu et al. 2019a). This type of paired sequential study where a laboratory-based set of experiments is then taken into the field to deepen understanding and flag any translational issues at an early stage should be encouraged as standard practice for the testing of novel candidate vaccines.

The duration of immunity is another factor that is infrequently taken into account in laboratory trials of novel vaccines; yet it is critically important in the field. Santos et al. assessed the impact of vaccine choice and different prime-boost regimens on duration of protective immunity in commercially reared turkeys housed together in an open-floor barn designed to mimic farm conditions. They found that although both the alphavirus-based and the inactivated reverse engineered vaccines induced protection at 6 weeks of age (3 weeks post-boost), there were significant differences in protection at 16 weeks of age, with the best protection elicited by an alphavirus prime and inactivated vaccine boost (Santos et al. 2017). Bertran et al. also identified the potential for long lasting immunity to be induced by a reverse-genetics inactivated H5N1 vaccine, which completely protected SPF white leghorn hens from challenge at 20 weeks post-boost (Bertran et al. 2017); similarly, Ladman *et al.* showed that another alphavirus-based HPAI vaccine induced antibody responses and protection that endured to 18 weeks post-vaccination (Ladman et al. 2019). Remarkably, Bhatia et al. reported protection elicited by a high dose H5N1 challenge following a single immunization with a reverse genetics-derived live attenuated H5N2 vaccine at 200 days post-vaccination (Bhatia et al. 2016). Together, these studies

highlight the importance of assessing vaccine combinations with the potential not only for gains, but for losses of potential immunity by sub-optimal regimens. The duration of protective immunity should be a standard parameter required/expected of all studies of novel vaccines, protocols and adjuvants as it has the potential for significant impact under field conditions.

In the field, poultry may be infected with pathogens at the point of immunization, but the impact of this on vaccine efficacy has rarely been studied in any detail. Spackman et al. used SPF chickens to study this phenomenon, finding that exposure of day-old chicks to the immunosuppressive infectious bursal disease virus (IBDV) significantly inhibited AIV antibody generation following vaccination 2 weeks later with an inactivated H7N3 vaccine (Spackman, Stephens, and Pantin-Jackwood 2018a). When previously IBDV-infected were challenged with homologous HPAI, they suffered mortality rates as high as nonvaccinated chickens, while chickens vaccinated in the absence of prior IBDV infection had 100% survival (Spackman, Stephens, and Pantin-Jackwood 2018a). Similarly, the use of the live NDV vaccine, LaSota, in the period around experimental infection of commercially reared chickens with LPAI H9N2 increased clinical signs, mortality, pathology and viral shedding (Ellakany et al. 2018a; Spackman, Stephens, and Pantin-Jackwood 2018b). When previously-IBDV-infected were challenged with homologous HPAI, they suffered mortality rates as high as nonvaccinated chickens, while chickens vaccinated in the absence of prior IBDV infection had 100% survival (Spackman, Stephens, and Pantin-Jackwood 2018a). Similarly, the use of the live NDV vaccine, LaSota, in the period around experimental infection of commercially reared chickens with LPAI H9N2 increased clinical signs, mortality, pathology and viral shedding (Ellakany et al. 2018b).

Commercial breeding hens typically pass down anti-AI antibodies to their progeny; therefore, any vaccine designed for use in the hatchery must be able to function well in the presence of maternally-derived passive immunity. Whilst previous studies have indicated an inhibitory effect of MDA on vaccine responses in chicks (Maas et al. 2011; de Vriese et al. 2010) it may be that this effect depends on the vaccination strategy used. Bertran et al. found that the presence of maternal antibodies to H5 AI did not interfere with responses of day-old chicks to a recombinant turkey herpesvirus (HVT) vaccine with an H5 AIV insert but impeded responses to an NDV-vectored H5 vaccine (Bertran et al. 2018); the latter since being confirmed by Murr *et al.* in the case of one or two week old chicks, but not in chicks three weeks of age or older (Murr et al. 2020). Further complicating the issue, there is some evidence that pre-existing MDA increased protection against AI elicited by a turkey herpesvirus-vectored H5N1 vaccine (Gardin et al. 2016). Therefore, at present, our understanding of the

mechanisms underpinning MDA-meditated enhancement or repression of vaccine-induced immunity is incomplete.

Modelling/Outbreak control

Effective vaccination has the potential to contribute to outbreak control by reducing transmission, enabling reduced culling of livestock and decreasing economic and psychological impacts for humans. The introduction of a bivalent H5/H7 vaccine in the Guangdong and Guangxi provinces in China between 2017-2018 in response to an HPAI outbreak led to a 92% reduction in detection of H7 viral RNA in samples from LPMs and reduced human infections with H7N9 by 98% in the region (J. Wu et al. 2019b). However, vaccine responses are also a major factor driving the emergence of new antigenic variants within the AIV population (Milani et al. 2017)(Y. Wu et al. 2021b). Therefore, understanding the right vaccine(s) to use at the right time and in the right place has been a key objective for modelling studies to date.

Even when vaccines are available, they are not always used due to concerns about cost-effectiveness, the ensuing trade restrictions, and their likely impact on control or surveillance outcomes; one example of this was the decision not to vaccinate during the 2015-2016 H5N6 and 2016-2017 H5N8 outbreaks of HPAI in France. Hautefeuillle et al. have since attempted to model the probable effects of vaccination during future outbreaks in the French poultry sector, aiming to establish the most impactful and cost-effective means of applying existing vaccines (Peyre et al. 2016). Using EVACS (Evaluation tool of VACcination Strategies), which was developed to compare different strategies for HPAI vaccine application in Egypt's poultry industry (Hautefeuille et al. 2020), the authors first modelled the French poultry farming network and likely routes of viral spread (based on data from the 2015/16 and 2016/17 outbreaks); when they applied various methods of vaccine use, they found that hatchery vaccination with a recombinant vector vaccine (where available for the species) of day old chicks would achieve the highest population-level of immunity and also had the highest benefit-to-cost ratio (Hautefeuille et al. 2020) Interestingly, although both French outbreaks have been concentrated in duck production units, the EVACS tool indicated that vaccination of ducks alone – or of other "high-risk" populations, such as free-range chickens - would likely be insufficient to prevent another outbreak (Z. Hu et al. 2020). These important data show that effective prophylactic vaccination strategies should focus on breadth of coverage across the sector, rather than depth of coverage in the at-risk group(s); they also strengthen the call for the development of vaccines able to induce effective and long-lasting responses in day-old chicks across the full range of commercial poultry species.

Molecular modelling can also be informative when considering optimal control strategies for future outbreaks. A fascinating approach was used by Metwally et al. who rationalised that the functional biological constraints on IAV HA meant that there were a limited number of variations that could arise which would enable the virus to remain infectious whilst attempting to escape the immune system of its host. Therefore, if one could model those variations and screen for them in existing vaccine seed strains, it should be possible to define an effective "cocktail" of vaccines to use within a region that would protect from circulating strains within a given subtype (Metwally and Yousif 2017). Through retrospective analysis of H5N1 outbreaks in Egypt in 2010-2014, for which data on viral sequence and vaccine usage were available, the researchers confirmed their hypothesis and were able to predict which vaccines could have provided additional protection against the AI field isolates (Metwally and Yousif 2017). The application of this type of molecular modelling approach for vaccine selection is further supported by findings that genetic variation within the HA1 head domain of H5N1 HA better predict antigenic variation than overall HA genetic difference; genetic variation between heterologous H5N1 viruses in these same regions of antigenic significance also correlated with cross-clade protection *in vivo* in vaccinated chickens (Peeters et al. 2017). However, these strategies are yet to be tested in the field under outbreak conditions.

Correlates of vaccine-induced protection

While neutralising antibody titre and HI have long served as the standard measure of the efficacy of immune response induced by inactivated AI vaccines, they do not, in fact, always accurately predict protection from disease. Zhao et al. found that immunisation of chickens with inactivated or Newcastle disease virus-vectored H7N9 induced low titres of antibodies capable of HI or viral neutralisation *in vitro*, yet they found high levels of non-neutralising IgG and full clinical protection of all chickens from H7N9 challenge (Criado et al. 2020). The researchers suggested that total anti-viral antibody levels may provide a more accurate correlate of protection, at least in the case of H7N9 immunity.

Petri et al. provide further evidence that predicting the extent of vaccine-induced protection and its impact on transmission remains challenging. They immunized groups of SPF chickens with one of two inactivated H5N1 AI vaccines and found that while immunization with the Karangganya strain could protect against Sukabumi strain challenge, the reverse did not apply, despite the same antigenic unrelatedness between the vaccine and challenge viruses used (Poetri et al. 2017). The researchers

highlighted additional key factors including the vaccine potency and strain antigenicity as having a major influence in determining future cross protection.

In the case of live inactivated AI vaccines, protection seems to correlate with the ability of the vaccine to induce elevated expression of interferon-stimulated genes in the trachea following intranasal administration of an NS1-truncated live attenuated vaccine derived from H7N3 (Jang, Ngunjiri, and Lee 2016a). How this innate gene expression connects with other immune factors in driving protection is yet to be investigated.

Interesting data have also emerged from retrospective analyses of situations in which vaccine have failed to perform as hoped in the field. Swayne et al. investigated the high rates of vaccine failure seen in Indonesian H5N1 outbreaks since 2005; they detected significant antigenic drift away from the vaccine seed strains as a primary factor, but noted that in most cases HI titre to the challenge strain correlated well with subsequent vaccine-induced protection from that strain, but not from antigenically-dissimilar strains (Swayne et al. 2015). The researchers also noted that the relationship between HI titre and protection may well vary by species, and even by breed, and that the investigation of non-HI factors to cross-protection required further investigation. Indeed, a more recent study on the vaccination of backyard poultry with an inactivated H5N1 vaccine revealed significant differences in response between the four avian species tested (Kandeil et al. 2017).

Cross-protection

The ability of a vaccine to induce protection against a range of influenza virus variants and strains is of paramount importance in the field, especially in the face of the antigenic drift that can occur in outbreak settings. In a wide-reaching study, Criado et al. conducted a set of vaccine-challenge experiments in chickens aiming to assess the level of cross-protection given by immunization with one of six inactivated influenza vaccines from the human pandemic preparedness program against eight homologous and heterologous isolates of HPAI H5N1 A/goose/Guangdong/1996 (Gs/GD) (Criado et al. 2020). While the levels of clinical protection varied between vaccine-challenge pairs as expected, almost all vaccinated birds exhibited significantly reduced viral shedding (Criado et al. 2020). Intriguingly, antibody titers prior to challenge did not predict protection, and some birds were partially protected from heterologous challenge despite the absence of detectable neutralizing antibodies to the challenge virus (Nassif et al. 2020). The researchers also identified a conserved pattern of epitope alterations within the HA protein that affected protection from all the vaccines tested, arguing for engineering of these epitopes in future vaccine strains.

Current vaccines have also been tested to assess their levels of cross-protection, although often not in field-like conditions. For example, Nassif et al. found that the commercially available turkey herpes virus-vectored recombinant HPAI H5N1 vaccine (Vectormune® AI) was able to clinically protect 80-90% of SPF chicks vaccinated subcutaneously at 1 day of age from intra-nasal challenge 28 days later with a homologous virus, or with H5N2 or H5N8 field isolates (Nassif et al. 2020). The researchers also found significant reductions in the frequency of birds shedding, and in the amount of virus shed, which is important for preventing transmission. A possible method to increase the cross-protective efficacy of current inactivated vaccines may be to boost immunity to the conserved M2e protein. There are promising initial data on this strategy from a study by Song et al., which showed that while chickens immunized with VLPs presenting M2e were not protected from either homologous or heterologous challenge, those primed with inactivated H5N1 and then boosted with the M2e VLP were completely protected after inoculation with either homologous or heterologous AI (B. M. Song et al. 2016). However, it is not clear whether this cross-protection would have been similarly induced by two immunizations with the inactivated vaccine, which was not tested in the study. Similarly, building on their previous work showing the potential of a chimeric norovirus P particle containing M2e (M2eP) to increase the breadth (but not strength) of response elicited by conventional inactivated vaccine (Elaish et al. 2017), Ghorbani *et al.* more recently assessed M2eP in combination with an H7N3 LPAI live-attenuated vaccine to understand whether combining these two approaches could induce broad protective immunity in chickens (Ghorbani et al. 2019a). They found that multiple M2eP boosts following a live-attenuated prime did transiently enhance protective immunity to heterosubtypic LPAI challenge, but that further study would be needed to understand the mechanisms of immunity important for effective cross-protection in this system (Ghorbani et al. 2019b).

Another method of inducing cross-protective immune responses is via the use of multi-epitope vaccines, in which the included epitopes are derived from heterologous influenza strains. Yu et al. constructed a recombinant baculovirus containing a sequence of cytotoxic T-cell, T helper-cell and B-cell epitopes from H1HA, H9HA and H7HA subtypes, and used it to intra-perintoneally vaccinate commercially reared chickens and SPF mice and assess their specific immune responses (Pushko et al. 2017). Encouragingly, both species mounted specific humoral and T cell-mediated responses to H7HA; however, these responses have yet to be linked to protection or cross-protection from challenge.

Extending the multi-epitope approach to the inclusion of whole multi-antigens, Pushko et al. generated a VLP that displayed H5, H7 and H9 HA, as well as N1 NA, and showed that chickens

receiving two doses of adjuvanted VLP vaccine mounted strong HI antibody responses. They also exhibited strong clinical protection from heterologous HPAI challenge and reduced viral shedding following HPAI or LPAI exposure (Kapczynski et al. 2016). Similarly, chickens immunised with VLPs containing HA from three distinct clades of H5N1 HPAI, as well as NA, mounted robust antibody responses and were protected from challenge with H5N1 and H5N8 AI (Ross et al. 2019).

Ross *et al.* harnessed computational modelling - using the Computationally Optimized Broadly Reactive Antigen (COBRA) approach - to generate a novel HA gene that was predicted to elicit broad cross-protective responses in chickens (Ross et al. 2019). They found that VLPs expressing the novel HA induced a greater breadth of cross-reactive antibodies capable of HI of diverse H5 strains, and protected chickens from challenge with an antigenically-drifted H5 clade virus, while VLPs expressing native H5 HA did not (J. E. Lee et al. 2020). The data from these studies further strengthen the case for VLP use as the basis of cross-protective vaccine design. For more on novel VLP-based vaccines see here.

Adjuvants

Adjuvants have traditionally been considered only for their ability to increase the size of response to a vaccine antigen, though we now know that they can also affect the type and breadth of immune response elicited. Several studies have identified novel adjuvants capable of increasing the magnitude of immune response to AIV vaccines. For example, using *Bacillus subtilis* spores with inactivated LPAI H9N2 increases immune responses in chickens (Gan et al. 2019), as does the incorporation of recombinant chicken IFNα (P. Gu et al. 2020), while polyethylenimine-coated PLGA nanoparticle-encapsulated Angelica sinensis polysaccharide outperformed conventional oil- and alum- based adjuvants as an immune stimulant in chickens immunized with an inactivated H9N2 vaccine (Tang et al. 2019). The effects of these adjuvants on the broader immune response and, in some cases, protection, have yet to be established.

As several novel vaccine candidates make use of the intra-nasal route of administration, there is a need for testing of mucosal vaccine adjuvants. Yitbarek *et al.* showed that Type IIb *Escherichia coli* heat-labile enterotoxin, when fused to H5 from HPAI H5N1 and instilled intranasally, was strongly immunogenic in mice and in chickens, where it elicited high titres of antiviral IgY, IgA and HI and neutralising antibodies in serum (Alexander Yitbarek et al. 2019).

Specifically aiming to enhance the immune response of ducks to conventional inactivated AI vaccines, Zhang *et al*. used the TLR3 ligand Poly I:C (which mimics the effects of viral double-stranded RNA on cells) as an adjuvant for an H9N2 vaccine in commercially reared two-week-old ducks (A. Zhang et al. 2017). Compared to ducks vaccinated with inactivated H9N2 alone, ducks that received Poly I:C/H9N2 mounted high anti-influenza antibody titres and in a shorter time, as well as higher levels of IFN-α, IFN-γ, IL-6 and MHC-II mRNA in cells from spleen; moreover, they exhibited reduced virus shedding after challenge with an antigenically-drifted H9N2 virus (A. Zhang et al. 2017).

Lone *et al*. compared a panel of 10 different adjuvants encompassing synthetic oil-based, plant oil-based, synthetic polymers, natural carbohydrates and seaweed-derived alginate for their ability to enhance immune responses to beta-propriolactone (BPL)- or formalin- inactivated H7N3 LPAI in 1-month-old SPF chickens (Lone, Spackman, and Kapczynski 2017). The authors found that almost all adjuvants induced higher levels of antibodies to the vaccine virus compared to nonadjuvanted controls, and greater clinical protection from homologous HPAI challenge; however, BPL-inactivation was clearly superior to formalin-inactivation methods, leading to greater immunogenicity. They concluded that mineral- or plant oil-based adjuvants were optimal for injected whole inactivated AI vaccines, and that BPL-inactivation should become the method of choice (Lone, Spackman, and Kapczynski 2017).

In line with the theory of Sitaras *et al.* (Sitaras et al. 2016) that achieving cross-protection is at least in part simply down to stimulating a strong enough vaccine-induced response to a partially-related AI strain, Lu *et al.* showed that combining the immunopotentiator CVCVA5 with conventional oil-in-water adjuvanted inactivated H5 vaccine stimulated increased antibody and cellular immune responses to the vaccine across a range of poultry species, which was associated with improved antibody cross-neutralization *in vitro* compared to vaccine alone (J. Lu et al. 2016). Whether this response translates into increased protection *in vivo* remains to be established.

Dietary interventions to improve vaccine responses

Given the findings on the immune-modifying effects of the intestinal microbiota in birds (see here), it is perhaps unsurprising that studies are starting to show that the intestinal microbiota also affect responses to AI vaccination. One recent study compared the post-vaccination immune responses of SPF chicks that were pre-treated with probiotics, antibiotics, or by faecal microbial transplant (Alexander Yitbarek et al. 2019). The researchers found that antibiotic treatment was associated with lower cellular and humoral responses to inactivated H9N2 vaccine, while probiotic treatment was

associated with higher virus-specific antibody titres; importantly, faecal microbial transplant could rescue the effect of antibiotic treatment, supporting the direct association between the gut microbiota and vaccine responses (Nofouzi et al. 2021). Manafi et al. also studied the effects of supplementing the diet of commercially reared chicks with either *Bacillus*- or *Lactobacillus*-containing probiotics on their responses to IV vaccination at day 7 of age (M. Manafi, Hedayati, and Mirzaie 2018). Intriguingly, while the highest dose of *Bacillus*-containing probiotics resulted in significantly higher HI titres on day 42 post-immunization compared to non-supplemented vaccinated controls, lower doses or treatment with *Lactobacillus* species was associated with lower than control levels of HI antibody (M. Manafi, Hedayati, and Mirzaie 2018). Given the broad effects of the gut microbiota on immunity, it is perhaps not surprising that the addition of the "wrong" bacteria could be deleterious to the response to vaccination. Defining the optimal probiotic supplement may require considerable research efforts, and tests under field conditions will be important to achieve the potential of this promising intervention.

A potentially easy way to improve vaccine responses would be to exploit the immune-modulating effects of natural feed supplements. Multiple studies have reported enhanced responses to conventional whole inactivated virus vaccines through oral administration of: heat-killed *Tsukamurella inchonensis* (Zakaria and Ata 2020), enzymatic treated soya protein concentrates (Harlystiarini et al. 2020), black soldier fly larvae meal (Shojadoost et al. 2020), selenium (Attia, Al-Harthi, and Abo El-Maaty 2020), fish- or plant-derived oils (Saleh et al. 2020), the plant flavonoids genistein and hesperidin (Kamboh et al. 2018), organic minerals (L. Lu et al. 2019), chromium (Jin et al. 2018; Zost et al. 2017), or golden needle mushrooms stems (Mahfuz et al. 2018). The mechanisms, and in many cases the relationship between enhanced response and protection, remain unknown. It would be interesting to understand how/whether these supplements affect the chicken gut microbiota and if this in turn relates to their immune-modulating properties.

Commercial and domestic poultry may be immunosuppressed as a result of viral infection, fungal contamination of their food, and/or stress. This immunosuppression can adversely impact responses to vaccination (as reviewed in (Schat and Skinner 2013)). A single study also looked at the possibility of using orally-administered immune modulators to increase vaccine-induced immune responses in immunosuppressed chickens. SPF chickens given oral ginseng stem-leaf saponins before induced immune suppression by cyclophosphamide injection, exhibited recovered general immune-responsiveness and antibody responses to an inactivated AI vaccine (J. Yu, Shi, and Hu 2015).

Novel vaccines

One of the key requirements of novel vaccine technologies is to facilitate the rapid and timely production of new vaccines. A recent paper reported the use of a reverse-genomics platform to generate an inactivated AI vaccine targeting HA and NA from HPAI H5N1 from the dominant clade circulating in Vietnam between 2012 and 2014 as a proof-of-principle study demonstrating the ability of the technique to expedite production of effective AI vaccines (Hoang et al. 2020). The authors used a low pathogenicity PR8 H1N1 backbone combined with the gene sequences for the HA and NA from the isolate of interest. The plasmids encoding all eight gene segments were transfected into 293T cells then rescued virus was propagated in SPF embryonated chicken eggs, where serial passage attested to its genomic stability and continued lack of pathogenicity. This virus was then inactivated before inoculation into three-week-old SPF chickens, where it induced over 90% protection from challenge with AI from within the same clade, and approximately 80% cross-protection against AI from a different clade; though it should be noted that both figures were lower than those following immunisation with the commercial vaccine NAVET-Vifluvac (Hoang et al. 2020). Similarly, Boubk et al. showed that a reverse-genetics approach could be used to effectively translate HA and NA sequences from recently circulating HPAI H5N8 into an effective vaccine capable of inducing complete clinical protection from homologous challenge in SPF chickens (Kang et al. 2020). Reverse-genetics engineered vaccines also have the potential to improve the process of vaccine seed-strain matching to circulating viruses. Kang et al. recently used this approach to generate five H5 vaccine seed strains for the Korean AI emergency vaccine bank, showing that the resulting egg-derived inactivated vaccines induced 100% protection in SPF chickens even after a single 1/10 dose (Kang et al. 2020).

Whilst these studies represent a promising demonstration of the potential of reverse genetics for production of animal influenza vaccines, the continued reliance on propagation of virus in embryonated hens' eggs remains a rate-limiting-step.

Virus-like particles (VLP) functionally mimic the antigenic structure of native virions but do not carry any genetic material and are therefore safe and relatively easy to manufacture, circumventing the need for embryonated hen egg culture; moreover they can be rapidly modified to take account of emerging variants. A recent study assessed the efficacy in chickens of a Montanide-adjuvanted VLP vaccine comprising M1 and HA proteins from LPAI H6N1 expressed using a baculovirus system, finding that two sub-cutaneous immunizations were sufficient to induce complete clinical protection from homologous challenge and high antibody titres that were cross-reactive with a human H6N1 isolate, as well as with antigenically-drifted H6N1 isolates *in vitro* (Zhu et al. 2020). Excitingly, the VLP platform

may also be able to be used from cross-species vaccines without significant modification: Hu et al. showed that a VLP comprising HA, NA and M1 proteins from a human H7N9 elicited neutralising antibodies and *ex vivo* antigen-specific cytokine production in both mice and chickens (Tatár-Kis et al. 2019).

In a comparative study in chickens, a novel H9N2 VLP vaccine out-performed the conventional inactivated equivalent, inducing higher levels of neutralising antibody and lower levels of viral shedding following homologous challenge (Xin Li et al. 2017). Similar results were found previously using an H6N1-VLP, which was able to induce cross-reactive antibodies in mice that inhibited the replication of diverse H6N1 isolates *in vitro*, while the conventional inactivated H6N1 vaccine did not (J. R. Yang et al. 2016). The efficacy of VLPs and their adaptation to mucosal administration may be achieved by the incorporation of dendritic cell-targeting sequences, as shown in a combined Newcastle disease virus and AI VLP vaccine in chickens (X. Xu et al. 2020). Moreover, there is some evidence that VLP-based HPAI vaccines may be able to overcome the issues of limited immunogenicity of some conventional inactivated AI vaccines in ducks: studies have shown strong antibody responses (Qin et al. 2019), and clinical protection against homologous HPAI challenge (Tatár-Kis et al. 2019). VLPs can also be efficiently produced in plants, with a recent study estimating that one kilogram of *Nicotiana benthamiana* leaf material would be sufficient to produce between five and 30 thousand chicken doses of an AI VLP vaccine (Nerome et al. 2017), compared to approximately three vaccine doses per chicken egg using current methods. Similarly, silkworm pupae might represent another low cost relatively efficient means of AI VLP production (Shehata et al. 2020a; Nerome et al. 2015).

One criticism of VLPs is that the lack of genetic material renders them potentially less immunogenic than live viruses. Live-attenuated influenza vaccines aim to overcome this and *do* include viral genetic material but are typically attenuated by truncation of NS1. One effect of this truncation is the generation of increased amounts of Type I IFN in response to LAIV vaccination, which has been correlated with their protective efficacy against LPAI in chickens (Jang, Ngunjiri, and Lee 2016b). Recently, Ghorbani *et al.* identified rare mutations in NS1 and PB2 proteins that increased the interferon-stimulatory properties of a reverse-genetics derived H7N3 LAIV; engineering both mutations into a novel LAIV significantly increased innate immune stimulation and reduced viral shedding after heterologous LPAIV challenge (Ghorbani et al. 2020). However, LAIV produced in this way continue to rely on embryonated chickens' eggs and there is a level of risk of recombination with circulating field strains which has been shown to occur readily, at least in chickens experimentally infected with H4N6 and H9N2 (Xuyong Li et al. 2018). The risk of recombination may be partially

ameliorated by emerging strategies such as modifications to the HA and NS1 packaging signals within the attenuated vaccine strain, as demonstrated by Chen *et al.* (S. Chen et al. 2021).

DNA vaccines have several attractive features as candidates for next-generation influenza prophylactics: they are relatively inexpensive to produce, can be produced and modified rapidly and safely, have the potential to induce long-lived humoral and cellular immunity, and are heat-stable, removing the need for cold-chain storage. However, previous candidate influenza DNA vaccines have suffered from variable immunogenicity *in vivo*, leading to a plethora of studies aiming to define the optimal strategy for their use. One such study demonstrated high efficacy in turkeys of a single intra-muscular immunization of saponin-adjuvanted DNA vaccine incorporating the full HA gene from LPAI H9N2 (Shehata et al. 2020b). Researchers observed complete clinical protection and effective inhibition of viral shedding, followed by the development of high titres of neutralizing antibody from three weeks post-immunisation (Huo et al. 2019a). However, this study did not assess T cell immunity, nor the longevity of the response, which therefore remain to be established. Stachyra et al. also demonstrated that codon optimisation can be a powerful tool to increase the immunogenicity of AI DNA vaccines, but also noted high inter-individual variation in response level (Stachyra et al. 2016)

Additional methods of increasing the potency of DNA vaccines have also been trialled. For example, the use of the pCAGGS plasmid that uses the chicken β-actin promoter to drive antigen transcription, rather than the commonly used mammalian promotors (as reviewed in (Meunier, Chemaly, and Dory 2016)). The incorporation of IL-2- and IL-7- encoding sequences also significantly increased the immunogenicity and efficacy of a DNA vaccine against infectious bursal disease virus in chickens (Huo et al. 2019b) but is yet to be tested in an AI DNA vaccine.

RNA vaccines against H1N1 have recently been trialled for the first time in mice, with promising results (Vogel et al. 2018); however, to our knowledge the same technology has yet to be tested in avian species.

Efforts to improve the efficacy of live vectored viruses are also underway. Tsunekuni et al. reported an increase in clinical protection from HPAI H5N1 challenge from 70% to 100% with an associated reduction in frequency of shedding birds from 50% to 0% when a recombinant avian avulavirus expressing H5N1 HA was modified to include the 3’ and 5’ untranslated regions of the HA gene (Shirvani et al. 2020). Similarly, the selection of improved viral vectors may represent a significant forward step. For example, Shirvani et al. compared the previously studied recombinant avian

paramyxovirus-1 (Newcastle disease virus) expressing HPAI HA, to a novel avian paramyxovirus 3-vectored equivalent, which should replicate in a greater number of host organs (Shirvani et al. 2020). While either vaccine was able to induce complete clinical protection against HPAIV challenge, the avian paramyxovirus-vectored vaccine gave rise to higher levels of neutralizing antibodies (Criado et al. 2019). A potential problem with NDV-vectored AI vaccines is the interference of existing anti-NDV antibodies, which are common in commercial flocks due to a comprehensive NDV vaccination program. One way of overcoming this issue is the modification of the NDV vector to remove immunogenic sites: Kim et al. exchanged the ectodomains of the H5N1 HA-expressing NDV HN and F proteins for those of avian paramyxovirus-2, which share 41% and 35% sequence homology at the amino acid level, and showed that it was able to induce significant protection from HPAI H5N1 challenge two weeks after the second dose (S. H. Kim, Paldurai, and Samal 2017). Similarly, a prime boost regimen using a chimeric NDV vector that is antigenically distinct from NDV and expresses HA and NA, followed by a boost with the HA-alone-expressing NDV vector produced complete protection against both HPAI and NDV in commercially reared chickens

Criado et al. also reported success using a recombinant fowlpox vector to protect chickens against HPAI H7N3, but the vaccine showed little ability to induce cross-protective immunity and would require regular updating to maintain efficacy (L. Xu et al. 2020). HerpesVirus of Turkeys' (HVT) is another promising viral vector candidate, with one study in commercial broiler chicks achieving 100% clinical protection from H5N1 challenge and 80-100% reduction in viral shedding, one month after a single immunization of day old chicks with an recombinant HVT vaccine containing the HA gene from H5N1 AI at the same time as an HVT vaccine against NDV (Abd El-Hamid et al. 2018). Also in HVT, Esaki et al. demonstrated the importance of optimal promotor selection in the case of virally-vectored vaccines, identifying the superior HI titres induced by immunization with HVT expressing H5 HA under the control of the cytomegalovirus promotor rather that the chicken beta actin or CMV/Bac chimera promotors (Esaki et al. 2015).

Viral vector strategies have also been designed specifically for use in ducks, via the use of attenuated vaccine strains of duck enteritis virus (DEV). Sun et al. inserted the gene for HA from H9N2 into DEV and found that a single intra-muscular immunisation induced sterile immunity to homologous challenge four weeks later (Y. Sun et al. 2017). Assisting in the development of future DEV vaccines, Zou et al. demonstrated the utility of the CRISPR/Cas9 system for the rapid generation of effective AIV vaccines (Z. Zou et al. 2017).

Another advantage of recombinant viral vectors is the potential to modify their biochemical characteristics, for example, a NDV vector with increased thermostability that allowed maintenance of initial titre for three days at 37°C and relatively high titres for 7 days at this temperature, whilst demonstrating enhanced immunogenicity in chickens and mice, has recently been reported (H. Lei et al. 2020a)Such developments are critical for the application of vaccination programmes in low-resource settings where cold-chain storage is not possible or reliable, but again issues of pre-existing immunity to the viral vector backbone must be carefully assessed.

Lactococcus lactis vectored vaccines offer several potential advantages over conventional vaccines, not least their independence from eggs for production; they are also suitable for needle-free administration via the oral route, but may require multiple administrations to elicit strong immune responses (reviewed in (Azizpour et al. 2017)). Current poultry vaccines for HPAI H5N1 are clade specific, but greater cross-protection is highly desirable. Lei et al. assessed the ability of a recombinant *L. lactis* displaying a portion of HA from H1N1 A/Vietnam/1203/2004 to induce protection from cross-clade challenge, and found that a regime of six oral doses during the first month of life elicited significant systemic and mucosal antibody responses and complete clinical protection from lethal challenge with homologous or heterologous clades two weeks later (H. Lei et al. 2020b). This study builds on previous work by the same group and others, showing the potential for *L. lactis*-vectored oral influenza vaccines: Sha *et al.* used a modified *L. lactis* secreting an M1-HA2 fusion protein and induced strong mucosal and systemic immune responses in chickens that significantly reduced clinical signs of LPAI H9N2 infection (Sha et al. 2020); while Lahiri et al. showed that *L. lactis* expressing NA or M2e induced mucosal and systemic immune responses against these antigens, not only in the gut but also in the upper respiratory tract of intra-gastrically-immunised chickens (Lahiri, Sharif, and Mallick 2019). Increased strength of immune response to intra-nasally-administered *L. lactis*-vectored H5N1 HA has been achieved by the addition of heat-labile toxin B subunit – a mucosal adjuvant – leading to complete clinical protection from H5N1 challenge two weeks later, accompanied by strong specific Th1-biased cellular responses and high systemic and mucosal antibody responses, which were significantly less, or absent, without the use of the adjuvant (H. Lei, Peng, Jiao, et al. 2015). The potential of adjuvanted *L. lactis* as a vector for effective and cross-protective influenza vaccines may even extend to cross-species use: Lei et al. used a cholera-toxin-adjuvanted *L. lactis* expressing H1N1 NP and administered it orally to mice, resulting in strong humoral and cellular immunity and 80% cross-protection from H3N2 and H5N1 challenge (H. Lei, Peng, Ouyang, et al. 2015); similar results were found in ferrets immunized with an unadjuvanted *L. lactis* expressing HA from H5N1, which was

able to induce protective systemic and mucosal immune responses after intra-nasal delivery (Cox, Patriarca, and Treanor 2008; Cox and Hollister 2009).

Lactobacillus plantarum is another bacterial species showing promise as an AIV vaccine vector. Most recently, Li et al. constructed an *L. plantarum* expressing a fusion of NP and M2 from LPAI H9N2, with the *Salmonella typhimurium* flagellin protein FliC as a mucosal adjuvant, on the bacterial cell surface (Q. Y. Li et al. 2020). After three oral doses, SPF chicks were challenged with homologous AIV. The authors found that, compared to conventional inactivated vaccine, the recombinant *L. plantarum* vaccine elicited significantly higher levels of specific antibodies in the lung and of virus-specific $CD4^+$ and $CD8^+$ T cells in the spleen, which translated to low levels of virus in the lung and high levels of survival (Q. Y. Li et al. 2020) Similarly, Yang et al. found that *L. plantarum* expressing NP and M1 from H9N2, combined with a DC-targeting peptide, was an effective intra-nasal vaccine, capable of inducing high levels of specific antibodies both locally and systemically, as well as higher frequencies of $CD8^+$ T cells in the spleen compared to conventional inactivated vaccine; however, these effects required six doses and failed to control viral titre in the lung to the same level as conventional vaccine (W. T. Yang et al. 2017). Six oral doses of *L. plantarum* expressing HA2 from H9N2 LPAI on its surface was effective in inducing high HI titres in serum but was outperformed by conventional inactivated vaccine, however comparable levels of HA-specific IgA were present in lung of the two vaccinated groups, and accordingly, both provided comparable reductions in lung pathology and lung viral titre (Couture MM-J et al. 2010) Thus, an orally-administered non-egg-propagated bacterially-vectored AIV vaccine has the capacity to perform as well as conventional vaccine in a LPAI challenge model.

Recently, the bat influenza virus subtype H17N10 has been used as the backbone for the development of a new live vaccine prototype against HPAI H5N1 in chickens (Schön et al. 2020). This vaccine provided full protection against a lethal challenge infection in both juvenile and subadult chickens and in ferrets. Importantly, due to its use of the bat influenza backbone, this vaccine is unable to reassort with avian influenza viruses, raising its safety profile.

Advances in vaccine production

Rapidity of production and non-reliance on the use of embryonated chickens eggs are desirable directions for future vaccine production. Petiot et al. reported the development of a new duck-derived cell line, DuckCelt®-T17, and conducted a proof-of-principle study of its use for inactivated influenza vaccine preparation (Petiot et al. 2018). This cell line showed good adaptation to suspension culture in animal-component-free medium, supported the replication of 18 influenza strains of human, swine

or avian origin, and demonstrated encouraging yields and scalability (Petiot et al. 2018). Though the study's main focus was on methods of production for human vaccines, this technology might prove ideal for the production of AI vaccines in future, especially given the evidence of egg-adaptation of IVs undergoing multiple passage in chicken eggs. Thus, the development of species-specific platforms for vaccine production may be desirable. Importantly, however, the ability of these new cell lines to be used for reverse-genetics-derived influenzas has not yet been tested and warrants further investigation.

The methods used for inactivating whole IV particles can affect virion structure and therefore immunogenicity. A study by Astill et al. compared the effects of the three main virus inactivation methods: formaldehyde, gamma irradiation, and BPL, finding that SPF chicks immunized intra-muscularly at seven and 14 days of age mounted significantly higher antibody responses to AI when the vaccine was inactivated by BPL or gamma irradiation compared to formaldehyde (Astill, Alkie, et al. 2018). Dumard et al. provisionally investigated the use of high hydrostatic pressure to inactivate both human and avian IVs, finding that it had potential as a method that induced only minor and often reversible changes to the capsid structure with some retention of biological properties but without infectivity (Dumard et al. 2017).

Another major problem with egg-based vaccine production methods is the possibility of contamination of the vaccine stock by inoculation of already-dead embryonated eggs. Existing methods aiming to avoid this are manual candling at the production farm, but some embryos may also die between the farm and vaccine production unit, this is highly labour intensive, and this method fails to detect approximately 5% of unsuitable eggs. To overcome these issues Kimura et al developed an LED-based method for screening out unsuitable eggs, and demonstrated a 93% discrimination rate with great potential for automation and high-throughput analysis (Kimura et al. 2015).

DIVA

A major issue with current vaccines is that they do not allow the differentiation of infected versus vaccinated animals, hampering surveillance efforts. Several novel strategies have aimed to overcome this, including the use of VLPs. In theory, any vaccine employing only part of the AI virion should allow DIVA by detection of antibodies to those proteins not included in the vaccine; however, in practice, many vaccines use the most immunogenic parts of the virus in their formulation, making detection of antibodies to the less immunodominant components of AI more challenging. However, Noh et al. demonstrated that this strategy can be successful: they immunized chickens with a chimeric VLP

comprised of HA and M1 proteins from HPAI H5N1 and a chimeric Newcastle disease virus fusion protein and achieved 100% clinical protection with reduced viral shedding, plus successful DIVA by detection of anti-NP antibodies in vaccinated-infected but not vaccinated chickens using a commercially available NP ELISA (Hautefeuille et al. 2020). Oliveira Cavalcanti et al. also successfully achieved cross-clade protection using a single immunisation with an adjuvanted H5 antigen generated using the baculovirus system (VOLVAC®B.E.S.T AI + ND KV, Boehringer Ingelheim Vetmedica), and demonstrated clear DIVA through the detection of anti-NP antibodies in vaccinated-infected, but not vaccinated-uninfected chickens (Oliveira Cavalcanti et al. 2017).

Swine Influenza Vaccines

As swine influenza is not generally a notifiable disease, and causes morbidity rather than mortality, global vaccination strategies vary widely. Ten years ago in the US, over 95% of swine nursery sites vaccinated their animals, but over half of these also reported SIV infections (USDA 2017). Despite the licensing of additional LAIV and RNA based-alphavirus vectored subunit vaccines for use in the US, issues of poor antigenic match between commercial vaccines and circulating strains remain, leading to strong growth in the production of autogenous inactivated vaccines, which now represent approximately 50% of IAV-S vaccines sold in the US (Sandbulte et al. 2015). In Europe, vaccination is much less widely used, and only 5-10% of sows are immunized (Reeth, Vincent, and Lager 2016).

Debate is ongoing on the optimal approach to vaccination of swine against SIV: whole inactivated vaccines can be effective in protecting from homologous challenge, and, unlike LAIVs, do not risk reassortment with circulating field strains; however, in the face of heterologous challenge conventional vaccines may be linked with the occurrence of vaccine-associated enhanced respiratory disease (VAERD), and establishment of effective immunity may be hampered by the presence of MDA. Thus, there is ample room for improvement of existing vaccines against SIV.

Route of delivery

Conventional inactivated vaccines are most frequently administered intra-muscularly. This has several drawbacks: it requires the production and disposal of vast quantities of biohazardous sharps waste each year, vaccines must be administered by well-trained personnel, the process is highly labour-intensive, the injected tissue cannot enter the human food chain, and the intra-muscular route is

notoriously poor at inducing mucosal responses which contribute significantly to protection from SIV. Thus, the ideal vaccine administration route would be designed to overcome these issues.

Effects of maternal antibody

Maternal antibody interference to conventional whole inactivated vaccines is well documented (Markowska-Daniel, Pomorska-Mól, and Pejsak 2011; Kitikoon et al. 2006), and recent attempts to vaccinate MDA-bearing piglets within the first days of life have confirmed this, proving ineffective at preventing SIV infection in the field (Ryt-Hansen, Larsen, Kristensen, Krog, and Larsen 2019). However, a laboratory study in 14-day-old piglets given an intra-nasally-administered LAIV vaccine indicated induction of immunity regardless of the presence of MDA (Pyo, Hlasny, and Zhou 2015).

An alternative perspective considers how the power of MDA could be harnessed through sow immunization. Pardo *et al*. carried out a prospective longitudinal field study of 52 commercial breed-to-wean farms in the US aiming to compare the effects of different sow vaccination strategies and the impact of commercial versus autogenous vaccine use on IAV infection in piglets at the point of weaning (Fabian O. Chamba Pardo et al. 2021a). They found that all vaccination strategies were effective at reducing the incidence of IAV infection in weaning piglets on the farms, though not the frequency of infected farms, and did not detect any differences between the use of different whole virus inactivated vaccine products (Fabian O. Chamba Pardo et al. 2021b).

Vaccine-Associated Enhanced Respiratory Disease (VAERD)

An intriguing phenomenon is the rare but well-documented occurrence of disease enhancement in pigs after immunization against SIV. This has been found to occur after vaccination with whole inactivated SIV (Khurana et al. 2013), recombinant HA (Rajão et al. 2014), or NP delivered via virus replicon particles (Ricklin et al. 2017) followed by heterologous challenge, or after immunization with either M2e fusion proteins or a DNA vaccine encoding M2e-NP followed by inoculation with H1N1 (Heinen et al. 2002). Understanding VAERD has proven challenging, but several studies have begun to shed light on various aspects of the processes involved. For example, it appears that not only the antigen but the route of delivery may influence the risk of VAERD: Bernelin-Cottet *et al*. compared the intra-dermal and intra-muscular routes of delivery for CpG-adjuvanted conserved IAV protein antigens (M2e, NP and HA2) and found that while the intra-dermal route of delivery was associated with increased frequency, severity and duration of symptoms, the same vaccine administered intra-muscularly elicited a partially-protective response (Bernelin-Cottet et al. 2016). The type of adjuvant used may also play a role. Souza et al. found that intra-muscular administration of H1N1 adjuvanted

with oil-in-water emulsion induced marked VAERD upon H1N1 challenge, while the same antigen delivered in squalene-based nano-emulsion or gel format adjuvant did not (Souza et al. 2018). A previous study by the same group effectively excluded a role for either age or timing of infection relative to vaccination in determining the onset of VAERD (Souza et al. 2016).

Earlier studies linked VAERD in pigs to the presence of non-neutralising antibodies to HA2 (Khurana et al. 2013), to mismatch in NA sequence (Rajão et al. 2016); and showed the capacity for amelioration by inclusion of M2e protein in the WIV vaccine preparation (Kitikoon et al. 2009). Moreover, there is some evidence that VAERD-inducing antibodies can be passed down from the WIV-immunized sow to her piglets (Rajao et al. 2016). In mice immunized against a human H3N2 strain, VAERD was associated with the induction of pathologic Th17 responses (Maroof et al. 2014), but to our knowledge this latter phenomenon has yet to be explored in pigs.

Parallels have been drawn between VAERD in pigs and other occurrences of antibody-dependent enhancement of viral infection, as most notably seen during some natural secondary heterologous infections with dengue virus (reviewed in Kuczera et al. 2018), causing major challenges for the design of protective vaccines against dengue. In the case of dengue, studies have indicated important roles for T cells (Nikin-Beers and Ciupe 2018) and complement (Byrne and Talarico 2021) in driving severe disease during secondary infection. These possibilities have yet to be assessed in SIV infection.

Correlates of vaccine-induced protection

Studies using the single-cycle replicating LAIV vaccine, S-FLU, which is available in Europe, have been informative in identifying correlates of vaccine-induced protection. Morgan et al. showed that S-FLU administered intra-nasally by aerosol induced both local and systemic $CD4^+$ and $CD8^+$ T cell responses, but it was the local responses that correlated with protection from homologous challenge, which occurred in the absence of systemic neutralizing antibody (Morgan et al. 2016a).

A single study has noted that the frequency of IFNγ-producing T cells is not related to protection following intra-nasal immunization with a temperature-sensitive reverse-genetics derived LAIV, but may be a useful measure of intra-muscular adjuvant/WIV vaccine immunogenicity in pigs (Olson et al. 2017).

Cross-protection

As a result of the constantly changing heterogeneity of circulating SIV strains, a single conventional whole-inactivated virus vaccine may not not protect against challenge with antigenically distinct strains. Therefore, much research has focused on ways of achieving greater cross-protection with existing WIV vaccines through the use of modified regimes or formulations, and several novel vaccines have been designed with the specific aim of inducing increased breadth of protection.

A study by Van-Reeth *et al*. showed that a heterologous prime-boost regimen with oil-in-water adjuvanted WIV European H3N2 followed by American H3N2 induced solid protection against both strains, as well as cross-reactive antibody responses to further antigenically-distinct H3N2 isolates (Van Reeth et al. 2017). Interestingly this response could not be recapitulated by a single immunization with a bivalent vaccine containing the same WIV strains, however it is not clear whether boosting with the bivalent vaccine would have increased the cross-reactivity. The same group then went on to assess the efficacy of the same regimen in pigs with pre-existing immunity to human-origin H3N2, finding that prior influenza exposure increased and broadened the response to heterologous prime-boost immunization (Chepkwony et al. 2020). Similarly, a recent study comparing homologous and heterologous prime-boost strategies using multivalent commercially-available inactivated SIV vaccines and a bivalent commercially-available LAIV demonstrated potential for a heterologous approach: the authors found that any heterologous regimen conferred improved immunity to simultaneous H1N1 and H3N2 homologous challenge compared to homologous prime-boost (C. Li et al. 2020). Together, these studies pose important questions about the mechanisms of increased protection/cross-protection elicited by heterologous vaccination in swine and whether it is the nature of the prime and boost, the order in which they are applied, or simply the presence of any two heterologous vaccines that is important in this process.

The capacity of DNA vaccines to encode both conserved and strain-specific antigens may also be exploited to generate vaccines with broad cross-protective potential. Borggren *et al*. studied the immune response induced by two immunizations of swine with an α-tocopherol-based adjuvanted DNA vaccine encoding NP and M from one H1N1 strain, with HA and NA genes from a different strain of H1N1 and an H3N2 isolate (Borggren et al. 2016). The intra-dermally-administered DNA vaccine elicited antibody and T cell responses that recognised both homologous and heterologous viruses *in vitro*, although protective efficacy *in vivo* is yet to be established.

Adjuvants

Alongside the ability of adjuvants to increase the magnitude of the immune response, recent data also suggest that certain adjuvants may be able to broaden the antigen recognition elicited by conventional inactivated whole virus vaccines, with the potential for enhancing cross-protection. Dhakal *et al*. first reported the use of corn-derived alpha-D-glucan nanoparticles as a novel adjuvant for inactivated SIV administered intra-nasally, and showed encouraging induction of local cross-reactive specific IgA but without inducing cross-reactive T cells or serum IgG responses to the vaccine (Dhakal et al. 2019). However, a follow up study aiming to enhance systemic responses to the vaccine by incorporating Poly I:C, the potent immunostimulatory TLR3 ligand, into the nanoparticles along with either inactivated SIV or conserved SIV B cell and T cell peptide antigens showed minimal improvements (Renu et al. 2020).

Other adjuvants have also been trialled as enhancers of cross-protection by whole-inactivated SIV vaccines. Following their work showing that a Poly[di(sodium carboxylatoethylphenoxy)-phosphazene-adjuvanted inactivated H1N1 SIV vaccine induced strong local and systemic specific antibody and potentially-cross-reactive T cell responses that together protected from virulent H1N1 challenge (R. Magiri et al. 2018), Magiri *et al*. went on to establish whether this T cell response was able to cross-protect against H3N2 after intra-dermal immunization of swine: it was not (R. B. Magiri et al. 2020).

Invariant natural killer T (iNKT) cells have important roles in viral infections: upon ligation of their MHC-I-like CD1d molecule, iNKT secrete large amounts of T-cell stimulatory cytokines and support the activation of antigen-presenting, NK and B cells (reviewed in (Opasawatchai and Matangkasombut 2015)). Two studies have assessed the use of the iNKT cell ligand, αGalacosyl-Ceramide (αGal-Cer), as a novel adjuvant for SIV vaccines. Dwivedi *et al*. combined αGal-Cer with UV-inactivated H1N1 administered as intra-nasal drops; they found that the addition of αGalacosyl-Ceramide increased WIV-induced specific-IgA, NK cytotoxicity and IFNα levels in the lung, which was associated with significantly decreased viral titres and pathology following homologous challenge, compared to WIV alone (Dwivedi et al. 2016). In their study on the effect of αGal-Cer as an adjuvant for intra-muscularly administered UV-inactivated H1N1, Artiaga et al. similarly saw significant enhancements to the immune response: compared to non-adjuvanted antigen, αGal-Cer-adjuvanted H1N1 induced higher levels of systemic and lung HI antibodies, as well as SIV-specific IFNγ-secreting T cells in the blood that localised to the lung during homologous challenge; also in the lung the authors saw increased levels of iNKT cells during infection, which was associated with reduced viral replication and shedding

(Artiaga, Yang, Hackmann, et al. 2016). Thus, αGal-Cer appears to be a promising candidate adjuvant for SIV vaccines capable of enhancing immunity and harnessing this potent cell population that may contribute to protection. Further studies are needed to assess the effects of αGal-Cer on cross-protection and to understand the role of iNKT cells in natural and vaccine-induced immunity to SIV.

Novel vaccines

Current vaccines against swine influenza typically have long production times and may not accurately reflect the current strains in circulation by the time they come to be administered. This becomes an especially pressing issue in the case of the emergence of a zoonotic strain with pandemic potential, when our ability to respond rapidly to the threat can have significant benefits in controlling the disease.

DNA vaccines have generated much interest in the swine influenza field for three main reasons: they are quick and easy to produce and modify to match the circulating strain, are capable of inducing broadly cross-reactive T cell responses – which current whole inactivated vaccines are notoriously poor at doing, and they can be delivered rapidly in a needle-free way. However, they often lack the ability to induce the high levels of antibody that are typically thought to be required for protection. One possible way of increasing antibody generation by DNA vaccines is by including an APC-targeting sequence to direct the translated antigenic protein to the APC at the immunization site. Building on their previous work with a DNA vaccine encoding MHCII-targeted HA from H1N1 (PR8) (Grodeland et al. 2016), Grodeland *et al.* recently showed that H1N1 HA linked to a chemokine-receptor targeting protein (MIP1α) was able to induce stronger IgG2 antibody responses in swine in the field, alongside higher levels of cross-reactive T cell activation compared to vaccination with the same DNA vaccine minus the targeting sequence (Grodeland, Fossum, and Bogen 2020). It remains unclear whether the increased level of IgG2 would be sufficient for clinical and virological protection following challenge, but these data represent a promising step forwards in the rational design of effective DNA vaccines for swine.

As for avian species, the use of viral RNA sequences to stimulate specific immune responses in swine is largely unexplored. A single study has reported the results of an RNA-prime, inactivated boost strategy against foot-and-mouth disease virus, with RNA-vaccinated animals exhibiting higher levels of neutralising antibodies, more quickly, and greater specific T cell responses, compared to pigs receiving conventional vaccine alone (Borrego et al. 2017). Given the recent success of RNA vaccines

in humans against SARS-CoV-2 (Dolgin 2021), generation and testing of RNA vaccines for SIV should be considered a research priority.

Other live-virus-vectored vaccine strategies have also given encouraging results on their cross-protective ability. One particularly interesting study documented the use of a live mouse-adapted human H1N1 strain as an SIV vaccine in commercially-bred piglets: subsequent challenge with SIV H1N1 or H3N2 showed strong control of local viral replication by day five post-inoculation (though not at day three), in the absence of detectable increases in serum HI antibodies (Zhao Wang et al. 2019). The mechanism of protection was unclear, but the authors speculate that it may be attributed to the activities of non-HI antibodies, mucosal antibody or cell-mediated immunity.

Again with the aim of inducing strong cross-protective immunity in swine, McCormick et al. exploited the power of molecular breeding (also known as DNA shuffling) to recapitulate the effects of viral genome evolution *in vitro* and generated four chimeric HA gene sequences representing distinct H1N1 clades. An initial study showed that these proteins delivered on an inactivated reassortant influenza virus background were broadly immunogenic in pigs, but their protective efficacy was untested (McCormick et al. 2015). More recently, they conducted a challenge study in pigs and found that two intra-nasal immunizations with a live parainfluenzavirus-vectored vaccine expressing one of the chimeric HA antigens induced broadly cross-reactive antibodies in sera and strong protection from homologous H1N1 challenge (Z. Li et al. 2020). It will be interesting in future studies to establish the full breadth of protection elicited by this novel vaccine.

An important feature of any novel vaccine for use in young animals is that it be able to induce effective immunity in the presence of MDA. In 2017, the US licensed a bivalent LAIV incorporating two reverse-genetics-derived viruses (Ingelvac Provenza™). Intra-nasal immunization with this mixture of NS1-truncated H3N2 and the same virus but possessing H1N1 HA and NA, was able to induce long-lasting immunity that reduced the incidence and shedding of virus from H1N2 or H3N2 challenged piglets (Kaiser et al. 2019), and was effective regardless of the presence or absence of MDA (Genzow et al. 2018). The ability of this vaccine to induce equally effective immunity in MDA -positive or -negative piglets is a major step forwards in reducing the heterogeneity of the national/regional herd response to immunization; however, this was tempered by recent findings showing that the vaccine strain had likely reassorted with circulating field strains in the US as soon as 2018 (Sharma et al. 2020). A recent strategy reported the use of a chimeric bat influenza vectored LAIV expressing truncated NS1 as a strategy designed to prevent recombination while retaining immunogenicity (Jinhwa Lee et al. 2021);

however the vaccine was not assessed in the presence of MDA. The use of DNA vaccines does not carry the risk of genetic reassortment and may also circumvent the potentially-inhibitory effects of MDA upon vaccine-induced immune responses. Sisteré-Oró et al. recently demonstrated that an alpha-tocopherol adjuvanted DNA vaccine encoding HA antigens fused with the bacterial immune-activator flagellin induced cross-protective responses that significantly reduced viral shedding at days five and 7 post-challenge with H1N1 or H3N2, regardless of the presence of MDA (Sisteré-Oró et al. 2019b).

Use of computer modelling for vaccine design

The induction of cross-protective immunity is a major goal of swine influenza vaccine research. A novel approach to the design of cross-protective vaccines is the use of computer modelling to predict an optimal cocktail of vaccine antigens to maximise the epitope coverage of a diverse protein, such as influenza virus HA. This field has progressed rapidly in recent years: using their own PigMatrix platform (Gutiérrez et al. 2015), Gutierrez *et al*. identified class I and II SLA epitopes predicted to cover seven representative IAV-S strains and generated a multi-epitope DNA vaccine that induced high levels of epitope-specific T cell responses in commercially reared outbred piglets (Gutiérrez et al. 2016). Utilising the same computationally-designed DNA vaccine, Hewitt et al. experimented with various combinations of homologous or heterologous boost (using the bivalent FluSure XP® whole inactivated H1N1/H3N2 vaccine), with fascinating results: pigs primed with the DNA vaccine and boosted with inactivated vaccine exhibited the highest level of peptide-specific T cell responses *and* comparable humoral responses to animals receiving two immunizations with the inactivated vaccine following H1N1 challenge (Hewitt et al. 2019).

Antigenic relatedness of SIV strains is often estimated based on their sequence identity, however this does not have a linear relationship with their immunological relatedness. In particular, cross-protection in the absence of neutralising antibodies is thought to be mediated by T cells (Hiremath et al. 2016; Morgan et al. 2016b), making “T-cell antigenic relatedness” a key parameter. Based on data from the PigMatrix platform, Gutiérrez et al. went on to develop the EpiCC algorithm, which links the identified SLA-I and -II epitopes with their predicted T-cell-facing structures and thereby assesses the likelihood of their recognition by vaccine-elicited T cells (Gutiérrez et al. 2017). Concentrating on T cell epitopes within the highly-variable HA region, the authors were able to link epitope relatedness between vaccine and challenge strains to clinical efficacy based on data from previously published studies (Gutiérrez et al. 2017).(Hiremath et al. 2016; Morgan et al. 2016b), making “T-cell antigenic

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Another algorithm that is generating exciting new data to inform DNA vaccine design is Epigraph. (Theiler and Korber 2018). Bullard et al. recently used this tool to compute optimal H3 HA sequences for incorporation into replication-defective adenovirus vectors (Bullard et al. 2021). A single intra-muscular immunization of the viral-vectored Epigraph-designed antigens into outbred piglets generated cross-reactive antibodies to 11 of the 13 swine H3 strains tested, as well as to three of the seven human isolates of H3, and broadly-reactive T cell responses; neither of which were seen after a single immunization with commercial inactivated vaccine (Bullard et al. 2021). Cross reactivity to human and swine H3 was boosted in both novel and conventional vaccine groups by a second administration three weeks after the prime; however, the strong response induced by the Epigraph antigens in a short time frame is noteworthy and could provide a critical advantage in an outbreak or pandemic scenario.

Equine Influenza Vaccines

Vaccination remains a primary method of control of EIV outbreaks globally, but with disparate regulations governing its use in different countries and situations. The OIE now recommends that all EIV vaccines include both Florida Clade 1 and 2 viruses to increase protection (OIE 2019), but a close antigenic match between vaccine virus and circulating strains remains necessary for adequate protection: this in turn requires ongoing surveillance and sequencing of field strains. Furthermore, protection is also affected by the immunisation schedule, which is complicated further by the presence of MDA in young foals (reviewed in (Daly and Murcia 2018)).

Effects of maternal antibody

Defining the optimum timing and approach to vaccination is a key factor in any successful immunization campaign. A recent study detected evidence of strong interference from maternal antibodies in foals injected with an inactivated equine influenza at 3 days of age, which exhibited rapid declines in antibody titre from 3 weeks post-immunization, in contrast to foals from unvaccinated

mares (Tallmadge et al. 2017). Interestingly, despite their low levels of antibodies, foals from vaccinated mares exhibited comparable immunoglobulin class switching and antibody diversity, demonstrating that the effects of maternal antibody interference are likely to be restricted to limiting the duration of vaccine-induced immunity in this setting, rather than impacting the qualitative aspects of the antibody response.

Maternal antibody interference was also seen in a field vaccination study in foals using a canarypox-vectored EIV vaccine, which found that over 98% of foals vaccinated between 4 and 9 months of age were predicted to be unprotected at 3 months post-boost, likely as a result of MDA (Fougerolle et al. 2016a). Importantly, when the prime immunization was administered 7 weeks later in a separate cohort, significant increases in humoral immunity were detected in the foals at the same timepoints (Fougerolle et al. 2016b). Interestingly, whilst an earlier study also found that initial immunization with a multi-valent inactivated EIV-containing vaccine at 3 versus 6 months of age led to significantly lower specific antibody levels to EI, the researchers observed that several measures of cellular immunity were comparable between groups (Davis et al. 2015).

Studies of licensed vaccines

Several vaccines against EIV are commercially available, and a recent study has compared the responses to each in groups of young sport horses to establish the speed of antibody induction after a prime-boost program, and the duration of antibody titres predicted to provide clinical and virological protection. The authors found that a canarypox-vectored vaccine was slower to induce seroconversion compared to recombinant or subunit vaccination, which induced seroconversion in almost all horses by day 7 post-boost (Entenfellner et al. 2020a). Two months later, the antibody response to whole inactivated vaccine was high enough to be likely to protect over 90% of horses, compared to 62% and 48% of horses that would have been protected after immunization with the subunit and live-vectored vaccines respectively; however, by six months none of the vaccine responses remained high enough to be likely to achieve virological protection (Entenfellner et al. 2020b). This study highlights the need for significant improvements to EIV vaccines, with a particular focus on increasing the duration of immunity.

Due to regional or global regulations that require immunization of horses before travel, competition, or high-risk seasons for pathogen infection, many animals are given multiple vaccines concurrently, but it is unclear whether this affects the immune responses to the individual vaccines. An observational study by Ohta *et al*. showed that separating inactivated EIV vaccine boost from

immunization with a new live attenuated equine herpesvirus type-1 vaccination by two weeks led to far higher antibody responses to the influenza vaccine (Ohta et al. 2020). In contrast, there was no interference evident when inactivated EIV and inactivated equine herpesvirus-1/4 vaccines were co-administered to horses (Gildea et al. 2016). These findings call for further investigation and perhaps regulation of the equine vaccination schedule to avoid interference between vaccines, and also for further research in other species as to the effects of live and inactivated vaccine co-administration.

Accurate analysis of protection offered by current or novel vaccines inevitably requires experimental infection of the natural host. However, in the case of EIV, reduced pathogenicity of recently emerged FC2 strains has led to variable induction of clinical signs and the need for larger numbers of animals to be challenged in order to generate robust results. The most commonly used challenge method in these studies is room nebulisation, which was initially adopted due to its superior efficacy compared to other routes tested at the time, as well as being well-tolerated by the animals (Mumford, Hannant, and Jessett 1990), but a meta-analysis by Garrett *et al*. has now revealed that greater accuracy can be achieved by changing to individual nebulisation, particularly for less pathogenic EIV strains (Garrett et al. 2017). This is an important finding that makes clear evidence-based recommendations for a change to current protocols in line with the imperative for reduction and refinement of the use of animals in research.

Novel vaccines

Antigenic drift away from the vaccine seed strains currently used to produce inactivated whole virus EIV vaccines may limit their efficacy, and the generation of new seed strains and vaccine stocks is a slow and expensive process; moreover, immunity to conventional vaccines can be short-lived in horses (Entenfellner et al. 2020b). Thus, several novel vaccine candidates are under investigation. A recent study by Blanco-Lobo *et al*. described a novel bivalent live-attenuated reverse-genetics-derived EIV vaccine that aims to overcome these issues. Building on their previous work developing a monovalent H3N1 FC1 LAIV which was both safe and effective in horses (Rodriguez et al. 2018), the authors generated a recombinant virus vaccine containing the internal genes of an attenuated clade 1 H3N8 and the HA and NA of an attenuated clade 2 H3N8 EIV and tested it in horses (Blanco-Lobo et al. 2019a). Horses immunized twice, 29 days apart, with the novel vaccine by aerosol inhalation exhibited decreased clinical signs and lower levels of virus excretion than their non-immunized counterparts when challenged with virulent clade 1 or clade 2 virus 28 days later (Blanco-Lobo et al. 2019b). This represents the first live-attenuated bivalent vaccine effective against circulating clade 1 and 2 EIVs,

engineered onto a viral backbone that makes updates to the antigenic sequence relatively straightforward, and with the ability to be produced independent of egg culture. However, as duration of protection is a key shortfall of existing inactivated vaccines, this should be specifically assessed in future studies.

Modelling/Outbreak control

Whilst vaccines are commonly used in EIV endemic countries, some regions have not ever been affected by an EIV outbreak, such as New Zealand. Rosanowski *et al*. have developed epidemiologic (S. M. Rosanowski et al. 2016a) and combined epidemiologic-economic (Sarah M. Rosanowski et al. 2019a) models to predict and compare the efficacy and cost-effectiveness of different control strategies under specific outbreak scenarios in this country. They compared movement restrictions only with the same restrictions alongside suppressive (vaccination of all susceptible animals within a 3km radius of an infection site), protective (vaccination of animals in the zone between a 7 and 10 km radius ring from an infection site) or targeted (vaccination of animals on all breeding and racing properties within 20km of an infection site) vaccination strategies, finding that addition of suppressive vaccination offered the greatest reduction in cases, and that the speed of the vaccination response was a critical factor in successful control (S. M. Rosanowski et al. 2016b). An extension of the work to include economic parameters further supported the use of suppressive or protective vaccination versus movement restrictions alone, taking into account the cost of the vaccination programs themselves and the impact of whether the outbreak were to occur during or outside of the main breeding season, offset against the predicted cost of the ensuing outbreak (Sarah M. Rosanowski et al. 2019b).

Companion Animal Vaccines

Although rarely fatal, canine influenza has attracted growing research interest since 2015, when an avian-derived H3N2 originating from Korea/South China caused an outbreak in the USA (Voorhees et al. 2017; Watson, Bell, and Toohey-Kurth 2017), and proved transmissible to other companion animal species (Lyoo et al. 2015). Although human infection with CIV has yet to be reported, active surveillance and pro-active CIV control strategies are warranted.

Route of delivery

Conventional needle-based administrations have multiple limitations including the need for relatively high volumes of virus, a cold chain of storage, disposal of sharps (with associated safety and environmental issues), and the need for trained professionals for administration; furthermore, in the case of companion animals the use of needles may reduce compliance of owners not wishing to put their pet under stress. Initially developed with the aim of generating a thermo-stable format for human influenza vaccines with the possibility of self-administration that avoided the need to travel to a healthcare setting (Mistilis, Bommarius, and Prausnitz 2015), microneedle-coated patches have recently been trialled as an alternative administration method for CIV vaccines. Microneedle patches consist of an array of microscopic needles that are attached to a backing pad, which may be dissolvable or removable, and – alongside their practical advantages – are thought to effectively target antigen-presenting cells in the skin to induce potent immune responses with low doses of vaccine (for a recent review of this technology see (Rodgers et al. 2018)). Choi *et al*. showed that coating and drying and inactivated H3N2 CIV vaccine onto microneedle tips significantly increased the thermal stability of the vaccine at high temperatures and elicited strong HI antibody responses in guinea pigs (I. J. Choi et al. 2018). A subsequent study by the same group confirmed that microneedle delivery of H3N2 inactivated vaccine was as effective as intramuscular injection at inducing antibody responses in dogs (I. J. Choi et al. 2020).

An orally- delivered CIV would be highly desirable, but to date no reports have been published towards this aim.

Novel vaccines

The emergence of H3N2 CIV in the US in 2015 caused concern not only for the health of affected dogs, but also for other susceptible companion animals, such as cats, and about the risk of transmission to humans. While there was some evidence in mouse models that the existing vaccine against equine-origin H2N8 CIV may offer partial protection against the H3N2 strain (Willis et al. 2016), a new vaccine offering improved protection was needed. Researchers working at Merial Inc. developed a monovalent inactivated H3N2 vaccine soon after: after two immunizations puppies were challenged with virulent H3N2 and exhibited complete clinical protection coupled with an 80% reduction in viral shedding compared to the placebo-immunized group (Cureton et al. 2016). The availability and application of this vaccine has not, however, prevented increasing incidence of CIV outbreaks across the country (Voorhees et al. 2017).

Rodriguez *et al.* have since developed both monolvalent H3N2 (Rodriguez, Nogales, Reilly, et al. 2017) and bivalent H3N2 and H3N8 (Rodriguez, Nogales, Murcia, et al. 2017) LAIVs to protect dogs against CIV, with encouraging results in mouse challenge studies after single dose intra-nasal administration. However, these have yet to be proven effective in dogs.

Although many cats are routinely vaccinated against "feline influenza" which is the common name for flu-like symptoms caused by infection with feline calicivirus and feline herpesvirus, a CIV vaccine licensed for use in cats is currently lacking. Velineni *et al.* tested an inactivated CIV vaccine in ten kittens, showing its safety and effective induction of HI antibodies two weeks post-boost (Velineni et al. 2020). This proof-of-principle study showed that this vaccine could be used in a cross-species immunization program, though the protective efficacy in felines remains to be tested.

Ongoing research

Scientists at the US National Poultry Research Center are evaluating the efficacy of existing and novel AIV vaccines in poultry by conducting gene sequencing and evaluating serological cross-reaction among relevant isolates, and new viral vector-based vaccine approaches are also under study. In Germany, at Boehringer Ingelheim Animal Health, development of novel vaccines against H9N2 AIV is underway, and scientists at Erasmus MC Viroscience in Rotterdam in the Netherlands are working to design universal vaccine candidates against H5 AIV. At the University of Nottingham, meanwhile, researchers study the potential of plant-based vaccines against AIV, expressing avian IAV proteins in plants for the development of virus-like particle vaccines.

Research into swine influenza virus vaccination also continues apace. At the National Institute of Animal Health in Japan, scientists are working to establish criteria for SIV vaccine strain selection. In Argentina, at the National Institute of Agriculture Technology, researchers are developing antigen candidates by reverse genetics for swine IAV vaccines based on Argentinean isolates. Research continues at the Friedrich-Loeffler-Institut into novel platforms for swine IAV vaccines, including avenues for universal and DIVA vaccination methods; alongside, scientists there are developing and testing temperature-sensitive LAIV vaccines targeting SIV, in collaboration with the University of Freiburg and Ceva. The efficacy of heterologous prime-boost swine vaccination protocols are under study at the University of Minnesota, testing both live attenuated and multivalent inactivated vaccines against simultaneous infection of pigs with H1N1 and H3N2 SIVs. Also under study here are the impact

of vaccination on reassortment under field conditions, testing whether vaccines can reduce the likelihood of potentially hazardous antigenic shifts, and the use of pre-farrowing sow vaccination to consistently wean SIV-negative pigs. At Ghent University in Belgium, meanwhile, researchers are developing a sustainable vaccination strategy for cross-clade protection against H1N1 in both swine and humans.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of animal influenza vaccines should be considered priorities for future research:

- *better understanding of the mechanisms and immune-system-wide effects of adjuvants to enable rational design of improved vaccines with rapid onset of broadly-protective immunity*
- *multi-species vaccine platforms that are broadly cross-protective: a universal flu vaccine?*
- *design of vaccination strategies that take into account both potential protection and interference from MDA*
- *vaccine performance under field conditions/studies that validate laboratory models of vaccination by comparison to field conditions*
- *comparative studies of vaccine efficacy in avian species other than chickens, including domestic ducks and turkeys*
- *characterization of the post-vaccination adaptive cellular immune response in swine*
- *correlates/tests for vaccine protection in avian species and swine*
- *identification of factors that can affect the efficacy of AIV vaccination.*
- *assessment of novel vaccines according to their ability to prevent infection and transmission for prolonged periods post-immunization*
- *understanding the role of vaccines in driving escape mutations, and how to prevent them*
- *vaccines that can be mass-administered to poultry already placed on the farm, i.e. not* in ovo *or at hatchery*
- *vaccines to induce strong mucosal immunity able to block shedding of LPAI*
- *potent vaccines for less immunogenic flu subtypes (e.g. H9, H7)*
- *development of protective vaccines to prevent outbreaks of influenza in horses*
- *RNA vaccines*

Drugs/Therapeutic Approaches

Although a number of drugs are available for treatment of viral influenza in humans, no comparable therapeutic approaches exist for widespread use in animals, primarily because of the risk of the development of resistance. Some limited situations may be suitable for use of the existing drugs in animals, but for more widespread therapeutics, new agents must be developed that can be safely used in the huge numbers of livestock that are at risk of influenza, without resulting in novel drug-resistant strains of virus.

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps and priorities in animal influenza drugs/therapeutic approaches in 2014/15:

- *identify appropriate uses of antivirals in animals*
- *develop agents for widespread use without adverse effects on human health*
- *study dietary/environmental interventions influencing immunity to infection*
- *improve resistance to infection by selective breeding or genetic modification*
- *develop interventions to block virus replication*

Literature review

Human anti-influenza drugs for animal influenza

AIV in poultry is generally managed through prevention (via surveillance, biosecurity and vaccination) and eradication. Currently available drugs that are used for treatment of human influenza are not suited for widespread use in poultry. However, culling is not always an appropriate response to HPAIV infection in poultry or in endangered species of wild birds. To address the ongoing need for protection of specific birds from HPAIV, Twabela *et al*. assessed the effectiveness of the cap-dependent-endonuclease inhibitor baloxavir marboxil (Xofluza) and the NAI peramivir (Twabela et al. 2020). In chickens that were experimentally infected with H5N6 HPAIV, simultaneous treatment with baloxavir

marboxil resulted in full survival with massively reduced viral replication, whereas peramivir was somewhat less effective. Unfortunately, neither treatment was effective at preventing mortality if given 24 hours after infection, when clinical signs would first be expected, so the utility of this approach would depend on knowledge or suspicion of infection prior to clinical presentation. With early treatment, a dosage of 2.5 mg/kg was shown to provide protection to chickens and Pekin ducks for 48 hours.

Although NAIs are not approved for widespread use in poultry, their use in humans can nevertheless result in environmental contamination and exposure to wildlife. The active metabolite of oseltamivir is stable, and as it is not removed from waste water by conventional sewage treatment, it has been identified in river water at up to 865 ng/l. Similarly, levels of another NAI, zanamivir (Relenza) up to 59 ng/l have been measured in river water. Tepper et al. used a mallard model to demonstrate the emergence of zanamivir-resistance mutations in the N2-type H4N2 AIV in response to exposure to zanamivir in the water environment (Tepper et al. 2020). In common with previously observed zanamivir-resistance mutations in N1-type H1N1 LPAIV, the H4N2 mutations did not persist when the drug pressure was removed. By contrast, similar experiments have previously demonstrated the emergence of a persistent oseltamivir-resistance H1N1 mutation, suggesting that the likelihood of the development of resistance in response to NAIs in the environment might depend on both the drug and the genotypic prevalence, and is another factor that should be considered in relation to the choice of NAIs for human use.

Adamantanes were used for many years as anti-influenza drugs in humans and livestock, until the widespread development of viral resistance limited their effectiveness. To safeguard against the further establishment of resistance, which would restrict the potential utility of adamantanes in pandemic situations, animal products are monitored for the presence of adamantanes and their metabolites. A paper-based lateral-flow immunoassay has now been developed that can facilitate the inexpensive, point-of-use detection of five adamantanes (somantadine, rimantadine, amantadine, 1-adamantanemethanol and 1-adamantyl methyl ketone) in biological samples (Y. Gao et al. 2019).

Therapeutics for animal influenza

To limit the potential for the evolution of resistance to agents targeting individual viral proteins, gaps exist for the development of strategies for the stimulation of animals' immune responses to influenza viral infections. One approach that has been investigated in swine is the stimulation of host immune

responses by α-galactosylceramide, which is a superagonist of iNKT cells. Intranasal administration of α-galactosylceramide to piglets infected with H1N1 IAV resulted in amelioration of disease signs and restoration of weight gain, with reduction of viral titres in the respiratory tract and prevention of lung inflammation (Artiaga, Yang, Hutchinson, et al. 2016). Notably, however, prophylactic administration of α-galactosylceramide to pigs does not seem to protect them from subsequent IAV infection, even though it results in the systemic expansion of iNKT cells (W. Gu et al. 2021).

Interferons are antiviral cytokines with a range of biological activities, making them common targets for stimulation of expression in strategies to improve immune responses to infection. To facilitate the use of chicken IFNα as a therapeutic in poultry, it has been expressed in *Escherichia coli* as a thioredoxin-tagged fusion protein (J. Zhao et al. 2019). Following purification of the fusion protein and excision of the thioredoxin tag, the recombinant IFNα was shown in an *in ovo* H9N2 infection model to significantly reduce the viral haemagglutination titre and *HA* gene copy numbers, indicating that this system could be used for the scalable production of bioactive antiviral IFNα.

In addition to bioactive macromolecules, some trace-mineral micronutrients, such as selenium, are known to have beneficial effects on poultry health and performance, and have also been tested for potential antiviral activities. Growing yeast in media enriched with trace minerals concentrates these substances and increases their bioavailability via incorporation into organic compounds. In a comparison of the effects in chickens of dietary supplementation for 2 weeks with selenium-enriched yeast or with inorganic sodium selenite, challenge with H9N2 LPAIV resulted in significantly lower levels of cloacal shedding of virus in all selenium-treatment groups relative to untreated controls (Shojadoost et al. 2019). Notably, oropharyngeal viral shedding was significantly lower in chickens fed selenium-enriched yeast than in sodium-selenite-fed chickens or in selenium-free controls. Any of the selenium supplementation regimens resulted in elevation of expression of interferon-stimulated genes in caecal tonsils, and either a low dose of selenium-enriched yeast or a high dose of sodium selenite was associated with elevation of expression of *IFNA*, *IFNB* and *IFNG* genes in caecal tonsils and spleens. These results demonstrated the beneficial potential of selenium supplementation for control of AIV infections in poultry. A combined yeast-based dietary supplement containing zinc, selenium and chromium has also been assessed in broiler chickens (Sobhi et al. 2020). In addition to positive effects on growth rate, carcass traits and feed-conversion ratio, the supplement was associated with significantly higher HI antibody titres in response to vaccination against H5N1 AIV, as well as higher relative weights of bursa, thymus and spleen, compared with chickens fed a basal diet.

One approach for the prevention of IAV infection is to interfere with the initial host-cell-receptor binding. The sialylated human-milk oligosaccharides 3'-sialyllactose and 6'-sialyllactose have been assessed for antiviral activity against IAV (Pandey et al. 2018). In HI assays, 3'-sialyllactose was active against all 13 strains of IAV that were tested, although higher concentrations of 3'-sialyllactose were required for inhibition of H1N1 strains than for inhibition of H1N2, H3N2, H5N1, H5N8 and H9N2 viruses. To determine the *in vivo* effectiveness, 3'-sialyllactose was mixed with H9N2 AIV prior to intranasal challenge of SPF chickens, followed by a further 9 days of treatment with 3'-sialyllactose. Notably, in chickens challenged with 0.8 HAU of H9N2 AIV, co-treatment with 3'-sialyllactose resulted in the complete absence of virus in both oral and cloacal swabs throughout the study period, as well as the prevention of an anti-H9N2 antibody response, indicating that the virus was completely neutralised and excreted from the chickens. Although the 3'-sialyllactose treatment was not effective at neutralizing a higher dose of 8 HAU of H9N2 AIV, multimerization of 3'-sialyllactose has the potential to increase its active concentration and improve its usefulness for the prevention of H9N2 virus infection in chickens. Taishan *Pinus massoniana* pollen polysaccharides have also been shown to interfere with the entry of H9N2 AIV into chickens, resulting in significant, dose-dependent inhibition of viral replication (Shang et al. 2020). Pre-treatment of chickens with these polysaccharides enhanced disease resistance and delayed infection by H9N2 AIV.

Genetic modification

Although most of the research into therapeutic approaches to tackle IAV infections involves the oral or parenteral administration of exogenous agents, direct genetic modification of livestock also has considerable potential. For instance, the use of the Clustered Regularly Interspaced Short Palindromic Repeat activation-synergistic activation mediator system (CRISPRa-SAM) has been demonstrated for the enhancement of resistance to IAV in porcine cell lines (J. Jiang et al. 2019). Two antiviral genes were examined in this study: *Mx2*, which encodes a myxovirus resistance protein, and *B4galnt2*, which encodes β1,4 N-acetylgalactosaminyltransferase. The CRISPRa-SAM system enabled activation of these genes in the PK-15 and IPEC-J2 porcine cell lines. In PK-15 cells, activation of either gene resulted in elevation of antiviral activity. Compared with unmodified cells, haemagglutination assays demonstrated significantly lower virus titres as a result of infection with H9N2 IAV in cells with activation of *B4galnt2*.

The 3D8 single-chain variable fragment (scFv) is a mini-antibody with antiviral nuclease activity. Transgenic chickens that express the 3D8 scFv gene under the control of the chicken *ACTB* promoter have been generated by the use of recombinant lentiviruses (June Byun et al. 2017). One line of the

transgenic chickens was selected for viral challenge on the basis of a low yield of virus (in terms of plaque-forming units) from embryonic fibroblasts infected with H9N2 AIV. When directly challenged with H9N2 AIV, the transgenic chickens demonstrated a lower level of cloacal (but not oropharyngeal) viral shedding compared with the controls. Furthermore, following contact exposure, the transgenic chickens had significantly lower levels of viral shedding, lower incidence of seroconversion and significantly lower HI titres than the controls, suggesting a reduction in transmission between the transgenic birds. In another study of the antiviral effects of 3D8 scFv against H9N2 AIV, chickens were treated daily for 3 weeks with oral doses of *Lactobacillus paracasei* that had been engineered to express the mini-antibody (H. Choi et al. 2019). In each experimental group, some birds were then challenged directly with AIV, and among those that were infected by contact transmission, chickens that were fed 3D8-secreting *L. paracasei* shed significantly lower levels of virus than chickens that were fed 3D8-anchored *L. paracasei* or non-recombinant *L. paracasei*.

Transgenic chickens expressing constitutively active chicken IFN-induced protein with tetratricopeptides repeats 5 (chIFIT5) also showed marked clinical resistance and reduced viral shedding during infection with HPAI H5N1, though protection was not sterile and transgenic animals frequently exhibited developmental defects (Rohaim et al. 2018). The generation of duck RIG-I-transgenic chickens has been under patent since 2010 (US20110247091A), with the idea being further supported by recent data showing that overexpressed chicken interferon response factor (IRF) 7 could induce transcription from the duck RIG-I promotor in chicken DF-1 cells *in vitro* (Yanna Xiao et al. 2018).

Herbal products and botanicals

Herbal products and botanicals have great potential as alternatives to synthetic antiviral drugs. The complex mixtures of active substances that are present in the wide array of natural products already have many known therapeutic uses, and many more undoubtedly remain to be discovered. The potential benefits associated with these products include economical production, ease of incorporation into animal feeds, the low probability of the development of resistance, and possible additional contributions to animal health not specifically related to any antiviral properties. These antiviral properties typically fall into the categories of adjuvant effects or enhancement of immune responses in relation to vaccination, or enhancement of immune responses to viral challenge.

Antibiotic growth promoters (AGPs) have been widely used in poultry production to enhance growth parameters, prevent disease and stimulate immune responses, but their inappropriate use leads to the development of bacterial drug resistance. A plant-derived non-AGP (Natusol), which consists of phytomolecules, direct-fed microbials, glucamannan oligosaccharides and organic acids, has been directly compared with the AGP bacitracin methylene disalicylate for its effectiveness in commercial broiler chickens (Milad Manafi 2015). Compared with chickens that were fed only the basal diet, supplementation with either AGP or non-AGP resulted in significant improvements in growth parameters and antibody titres in response to standard vaccinations, including AIV. Similarly, a herbal compound consisting of thyme, oregano, chamomile and peppermint essential oils has been shown to perform as well as the AGP phospho-flavomycin with respect to growth parameters and post-vaccination AIV antibody titres (Hedayati and Manafi 2018).

In addition to plants, yeast and algae are sources of potentially beneficial antiviral compounds. Red algae contain carrageenans, which are high-molecular-weight sulphated polysaccharides that have been shown to have a number of antiviral properties. The effects of κ-carrageenan on infection and replication of swine pandemic 2009 H1N1 IAVs have been studied *in vitro* (Shao et al. 2015). The polysaccharide was found to dose-dependently inhibit replication of two pandemic H1N1 strains in MDCK cells, at concentrations that did not result in cytotoxicity. The results of HI assays demonstrated that κ-carrageenan specifically interacted with the HA protein of the two pandemic H1N1 strains, and did not affect the HA proteins of other swine H1N1 or avian H9N2 IAVs. The interaction of κ-carrageenan with HA interfered with viral adsorption to MDCK cells, and the polysaccharide also had the further effect of inhibition of IAV mRNA and protein expression following viral cellular internalization.

In chickens, dietary supplementation with a combination of vitamin E and a commercial yeast-extract product (Fetomune Plus) has been shown to significantly reduce the effects of direct challenge with H9N2 AIV (Awadin et al. 2019). Infected birds with dietary supplementation had significantly lower expression of duodenal IFNγ (on day 3 post-infection [p.i.]) and duodenal and splenic IL-6 (day 5 p.i.), with higher expression of duodenal IL-2 (days 3 and 5 p.i.), as well as significantly lower levels of virus shedding, higher HI titres, lower levels of clinical signs and mortality, and lower H9N2 staining intensity by immunohistochemistry, compared with infected chickens that did not receive supplementation.

Ongoing research

At the University of Minnesota College of Veterinary Medicine, scientists are testing viral interference as a means for controlling AIV within infected premises. Newcastle disease virus, for instance, stimulates IFN production that can protect birds against HPAI, and this mechanism may be applicable on a wider scale for limiting the spread of AIV on poultry farms.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of animal influenza drugs and therapeutics should be considered priorities for future research:

- *intervention products, such as antivirals that prevent escape mutants*
- *application of SARS-CoV-2-related advances in therapeutics to IAV*
- *assessment of combinations of agents that show individual promise*
- *scalable, economical introduction of beneficial agents into animal feeds*

Disinfectants

The stability of influenza viruses is sufficient to allow transmission via contact between animals and contaminated surfaces. Therefore, surface disinfectants play a vital role in the prevention and control of avian influenza outbreaks. Disinfection of materials and vehicles entering poultry farms reduces the chance of cross-property transmission. If an outbreak is detected, post-culling cleansing and disinfection can help ensure that the virus is not transmitted to the next batch of poultry to occupy the premises. Disinfection steps are included in many official/governmental outbreak control procedures (Gale et al. 2020), and proper implementation of these steps can significantly reduce transmission risk and viral maintenance period at a given location.

Incomplete or inefficient disinfection, however, can create a false sense of security and hamper outbreak prevention and control efforts. Some commonly-used disinfectants are less effective at low or high temperatures or in the presence of organic contaminants (common in the messy environment of a poultry farm). Additional concerns include breakdown of disinfectants (e.g. from long-term storage or exposure to sunlight) and environmental impact from particularly hazardous substances. The ideal disinfectant will be suitable for long-term storage, cost-effective, environment-friendly, and highly effective against influenza virus within a short exposure time. Research has been ongoing over the past five years to describe disinfection methods that meet some or all of these criteria.

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps in animal influenza disinfectants in 2014/15:

- *methods for efficient application of disinfectants with minimal environmental impact*
- *development of fast-degrading disinfectants*
- *continued research into cost-effective reagents with focus on ease-of-use*

Literature review

A recent study by Thammakarn *et al.* examined scallop shell powder, primarily composed of $CaCO_3$, for viral inactivation properties, finding that a 20% suspension inactivated AIV within three minutes even under harsh conditions (emulated sunlight exposure and repeated cycling between wet and dry

conditions) and was less sensitive to neutralization than the similar slaked lime (Thammakarn et al. 2015). Similarly, Ruenphet *et al.* tested the efficacy of slaked lime and fresh charcoal ash in both powder and liquid form against LPAI H7N1 virus. They found both solutions effective against the virus via alkalinity, with charcoal ash able to inactivate H7N1 in less than a minute. This held even in the presence of organic contaminants that can interfere with the activity of more common disinfectants like chlorine and quaternary ammonium compounds (Ruenphet et al. 2019). Slaked lime reached similar efficacy under a longer timetable of 10-30 minutes depending on the presence of organic materials. Both solutions were also found robust to lengthy storage ($\geq$ 8 weeks at room temperature), with charcoal ash slightly more stable over time.

Taking an alternative route for disinfection, Nishisaka-Nonaka *et al.* tested inactivation of IAV using UV-emitting LEDs, finding that 280nm light (UVC) caused 10-fold decrease in the infectivity of H1N1 at a fluence of 0.028 J/cm^2 (Nishisaka-Nonaka et al. 2018). Interestingly, this effect seemed subtype-dependent, with HPAI H5N1 more sensitive to UV inactivation than H1N1.

Meanwhile, Guan *et al.*, searching for a disinfectant suitable for pandemic control during winter months, tested various compounds for activity at -20°C. They found that at this low temperature, the standard disinfectants Virkon (2%) and Accel (6.25%) caused 6-log inactivation of H6N2 AIV when supplemented with 20% propylene glycol, 20% methanol, or 20% $CaCl_2$ (Jiewen Guan et al. 2015). Propylene glycol and methanol did not kill AIV by themselves at this temperature, but 20% $CaCl_2$ alone caused 5-log inactivation within 10 minutes (Guan et al. 2015).

Environmental conditions can play an important role in outbreak control by influencing the length of time that AIV remains stable on exposed surfaces. In a later study, Guan *et al.* tested the effect of relative humidity on environmental survival of AIV by measuring time to 10-fold infectivity reduction under different conditions (J. Guan, Chan, and VanderZaag 2017). They found that, at the relatively standard farm temperature of 23°C, raising the humidity from 25% to 55% reduced this time from approximately 1.5 days to 0.32 days for H9N2 AIV on porous surfaces, and from 7.8 to 0.05 days on non-porous surfaces. H6N2 AIV was generally more stable but still faced 10-fold activity loss within a day on non-porous surfaces at 55% humidity. These results recommend humidifiers in poultry buildings as a general infection-reduction strategy, but the authors note that AIV has been shown to survive longer in wet poultry faeces than dry – more research would be necessary to weigh the sum benefits of higher humidity on poultry farms (J. Guan, Chan, and VanderZaag 2017). Temperature may itself be a means for decontamination – research by Stephens & Spackman evaluated thermal

inactivation on LPAI and HPAI present in poultry litter, finding that both types were inactivated by 24hr at temperatures from 32.2-48.9°C (Stephens and Spackman 2017). Depending on the heating capabilities of specific poultry houses, temperature treatment may be used in combination with other disinfection protocols to reduce the time from depopulation to repopulation and minimize economic impacts of an outbreak.

Another potential route for general disinfection/outbreak control on poultry premises is air filtration. Ventilation is mainly an understudied area of influenza control research – studies of HPAI H5Nx outbreaks have shown that virus release from poultry farms is consistent with the possibility of airborne transmission and surface deposition around infected premises (Torremorell et al. 2016; Scoizec et al. 2018), but further research is needed to clarify the amount of virus disseminated from poultry houses and how this may vary between outbreaks.

HEPA filters are known to effectively filter virus particles, but the humidity, high rates of air exchange, and large concentration of particulates at most poultry farms would render them cost-inefficient in this environment (Leibler et al. 2017). Other ventilation technologies that have been tested on farms are primarily targeted toward odour containment rather than pathogen filtration. A related option is to treat existing filters with a disinfectant compound capable of inactivating airborne AIV. For this purpose, Ren *et al.* tested filters coated with a member of the N-halamine family, known for their antimicrobial activity but not previously tested on AIV (Ren et al. 2018). N-halamines are low-toxicity and do not release gaseous chlorine, increasing their environmental suitability. Here, N-halamine-coated filters (nonwoven fabric from N95 respirators) were found to trap and completely inactivate avian H1N1 virus in bioaerosols (Ren et al. 2018).

In addition to further research on effective disinfectants, extended outreach and public education will be necessary to increase understanding of the importance of cleansing and disinfection procedures. Cui et al., surveying risk perception in Chinese poultry farmers in the Jiangsu and Anhui provinces, found that fewer than 50% of respondents always carry out regular disinfection of premises and any vehicles or materials entering the poultry farm (Cui et al. 2019). Also important to study further is the degree to which certain outbreak control procedures may be unnecessary if disinfection is carefully performed. Gale et al., for instance, modelled HPAI H5N1 reinfection risk between poultry restocks in an enriched colony-caged layer poultry house, finding that the lengthy and costly process of dismantling complex machinery for thorough disinfection may not be necessary if the premises

themselves are thoroughly disinfected and at least 40-90 days pass between culling and restocking (Gale et al. 2020).

Ongoing research

At the US National Poultry Research Center, researchers are studying the environmental stability of AIV, including the efficacy of disinfection/inactivation protocols and their impact on virus detection. Meanwhile, Defra-funded research at the UK Animal and Plant Health Agency investigates environmental survival of various influenza subtypes with the aim of reducing outbreak cost burdens and enabling rapid reestablishment of trade links post-outbreak. At the University of Minnesota, researchers are developing methods for containing bioaerosols via electrostatic precipitator technology, which can be applied to intake and exhaust air systems in swine farms to mitigate aerosol transmission.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of animal influenza disinfection should be considered priorities for future research:

- *Decontamination procedures for caged/layer poultry houses*
- *Increasing understanding of virus survival in the environment (e.g. fomite contamination timeframes, temperature stability, variability between subtypes, etc.)*
- *Risk analysis of contaminated land/fomites upon restocking*
- *Role and necessary level of cleaning and disinfection required for different outbreaks*

Depopulation and Disposal

In the event of an outbreak of notifiable avian influenza, culling of affected animal populations is often the first step in outbreak control. National policies on reportable disease containment may stipulate that affected farms be quarantined, movement halted, and animals humanely euthanized. The resulting animal carcasses must then be safely and efficiently disposed of to prevent further spread of the disease, clearing the way for disinfection and resumption of animal production. Despite the relative simplicity of depopulation compared to the biological complexities of vaccines and antivirals, the process involves numerous economic, ethical, and environmental concerns. Depopulation is necessarily an extremely costly action, causing the loss of entire populations of poultry and swine with potentially devastating downstream economic effects. Such costs may incentivize farmers in regions with low biosecurity to avoid reporting infected animals to regulatory agencies, allowing outbreaks to spread unchecked. From an ethical perspective, humane euthanasia of large animal populations is challenging, and the impact of depopulation on the human operators themselves must also be considered. Finally, carcass disposal has the potential to release significant amounts of contaminants into the environment (including influenza virus in the case of improper disposal).

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps in animal influenza depopulation and epidemic control in 2014/15:

- *defining necessary extent of depopulation depending on outbreak factors (e.g. transmission rate) with aim of reducing economic impact*
- *improving euthanasia protocols for layer/caged birds*
- *modelling outbreak spread vs. culling radii to determine smallest effective radius*
- *more humane culling methods*
- *reducing environmental impact of carcass disposal*
- *addressing depopulation and disposal in resource-limited regions*

Literature review

Depopulation and disposal are vital components of effective influenza outbreak response, but they are also expensive and challenging to implement at scale (Pramuwidyatama, Hogeveen, and Saatkamp 2019). Culling in particular is an extremely costly process, and it is very difficult to accurately predict appropriate cut-offs for culling that maximize disease control while minimizing unnecessary economic losses (Mummert and Weiss 2017). Lee and colleagues, for example, developed a spatiotemporal compartmental model of control measures in context of the winter 2016/2017 South Korea HPAI epidemic, finding an optimal culling radius of 2.65km for towns with a viral reproductive number (correlated with farm density and number of duck farms) > 1 (Jonggul Lee, Ko, and Jung 2019). This is smaller than the Korean government-recommended radius of 3km, illustrating the potential for computational modelling-based approaches to minimize unnecessary losses during an outbreak.

Gassing remains one of the most common methods for mass poultry culling during an AIV outbreak due to its relatively inexpensive equipment requirements and minimal necessary personnel training (EFSA 2018). Research continues into methods that may improve on the efficiency or humaneness of gassing – Benson *et al.*, for instance, tested the deployment of compressed air foam systems for poultry depopulation during an AIV outbreak, finding them to efficiently euthanize birds in a large caged layer operation with a faster time-to-unconsciousness than CO_2 gassing (Benson et al. 2018). It is important to note, however, that ethical guidelines are commonly breached under outbreak situations, with consequences not only for the welfare of the animals to be culled but also for the workers who perform the depopulation. Park and colleagues conducted a survey of frontline livestock workers, finding a very high incidence (>75%) of extreme stress and PTSD-like symptoms. They stress the importance of implementing strict pre-operational education and training procedures and providing mental health care to frontline workers during and after outbreak-related culling (H. Park, Chun, and Joo 2020).

Disposal methods vary between regions, with varying degrees of efficiency and environmental impact. In Korea, for instance, on-farm burial is the most common method, but this can lead to the generation of contaminants like ammonium-N and chloride. Yoon *et al.* tested biochar as a permeable reactive barrier in combination with fast-growing poplar trees to soak up contaminants, finding that such a barrier decreased leachates released from buried swine carcasses (J. H. Yoon et al. 2017). Composting and rendering are also popular methods, but these often require the use of dedicated sites for bulk disposal. Jenner *et al.* stress the need for relationships between government agriculture/public health agencies and commercial composting/rendering facilities to be established during “peacetime” in

order to create functional plans and workflows rather than waiting until an outbreak has arrived (Jenner et al. 2020). From the standpoint of private actors, economic considerations come to the forefront when considering different disposal methods. Huang *et al.* conducted a 2015 survey of Chinese poultry farmers' willingness to accept compensation to practice safe disposal of chickens infected with HPAI; they found that only 75% and 60% of farmers in South and North China, respectively, would adopt safe disposal methods at the 2015 compensation rate of 10 yuan/bird. This number increased to 80% in both regions if compensation was increased by 40-90% (Huang, Wang, and Zuo 2017). Further research will be necessary to help thread the balance between up-front economic loss (e.g. due to compensation for culled birds) and downstream costs from poultry trade losses.

Ongoing research

Researchers at the University of Minnesota College of Veterinary Medicine are conducting studies to assess the risk of different mortality disposal strategies during an AIV outbreak, specifically addressing the risk of virus transmission during transport of animal carcasses from farms to off-site disposal locations (e.g. for carcass rendering, incineration, or burial in landfills). Such systems have remained relatively unchanged in the USA even after the major 2014-2015 HPAI H5 outbreak, requiring focused attention and evaluation to ensure proper biosecurity measures are in place ahead of the next outbreak. Study at U of M is also underway to assess high expansion nitrogen foam for humane mass depopulation of poultry and swine farms in the event of an outbreak.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following aspects of animal depopulation and disposal following influenza outbreaks should be considered priorities for future research:

- *Computational modelling studies to determine minimal effective depopulation radii during outbreaks of various subtypes*
- *Rapid and humane methods of depopulation for caged/layer poultry farms with large populations*

Personal Protective Equipment

Personal protective equipment (PPE) is the "last line of defence" between a person and a potential infection with zoonotic influenza. Although process and engineering controls aim to reduce the risk of exposure of workers, during outbreaks individuals may still be exposed repeatedly to high titres of virus, particularly during mass culling operations. The design of effective, easy-to-use and comfortable PPE underpins high compliance rates and correct use, maximising protection. Alongside, monitoring the protection afforded by various strategies in the field is an important part of the process of designing improved approaches.

Previously identified knowledge gaps

Previous reports (United States Department of Agriculture 2014; OFFLU 2014; European Food Safety Authority 2015) identified the following priority research knowledge gaps in PPE for use in animal influenza control settings in 2014/15:

- *improved respirators (ease-of-use, comfort, etc.) for working under difficult field conditions*
- *availability of approved drugs and vaccines for animal workers*

Literature review

The design of effective strategies to keep workers safe from zoonotic infections with animal influenza viruses during epidemic control relies on a thorough understanding of the risks and the assessment of previously used strategies. A study of the incidence of avian influenza among poultry farm depopulation workers in Korea during the 2016-2017 HPAI H5N6 outbreak demonstrated the efficacy of combined use of PPE (disposable coveralls, nitrile gloves, N95 particulate half- mask with two- strap design, unvented goggles, and boots) and prophylactic administration of oseltamivir from day one of exposure to seven days after last exposure (Ryu et al. 2018a). All workers were instructed to report any respiratory symptoms, and among the 4633 workers enrolled in the study 22 did exhibit clinical signs of viral respiratory infection, however none were due to H5N6 infection (Ryu et al. 2018b). This data is encouraging, however as the mean days of depopulation per worker were just 1.5 (range 1-15), it remains to be seen whether the 100% protection afforded by the combination of anti-viral drug use and PPE would withstand longer periods of exposure.(Ryu et al. 2018a). All workers were instructed to report any respiratory symptoms, and among the 4633 workers enrolled in the study 22 did exhibit clinical signs of viral respiratory infection, however none were due to H5N6 infection (Ryu

et al. 2018b). This data is encouraging, however as the mean days of depopulation per worker were just 1.5 (range 1-15), it remains to be seen whether the 100% protection afforded by the combination of anti-viral drug use and PPE would withstand longer periods of exposure.

Previous studies in the USA also support the conclusion that appropriately-used PPE is effective in preventing transmission of AI to workers involved in outbreak control. Olsen *et al.* recently reviewed data from 2014-17 AI H5 and H7 outbreaks in the US and found zero cases of human infection among 4,555 workers who were potentially exposed to the virus while wearing PPE (recommended level C: dermal protection suit e.g. Tyvek, goggles and air-purifying respiratory protection (United States Department of Agriculture 2006)) and without the use of anti-viral drug prophylaxis (Olsen et al. 2019). Remarkably, even without PPE use, transmission may be a rare event under certain conditions: a study of 164 cases of self-reported exposure to infected birds during HPAI H5 outbreaks in the USA in 2014/15 again revealed zero instances of laboratory-confirmed human infection (Arriola et al. 2015). However, neither of the studies conducted in the USA included duration of individual exposure of the at-risk workers.

Future research priorities

Based on the above literature review and with reference to previously identified knowledge gaps and expert opinion, the following areas of animal influenza vaccines should be considered priorities for future research:

- *improved respirators (ease-of-use, comfort, etc.) for working under difficult field conditions*

Conclusions

A huge number of studies have been published across the various fields of animal influenza research in the past six years: approximately three studies per day, every day. Some of these studies report major breakthroughs in new areas, begin to answer key questions or pave the way for future work with the promise of significant gains in knowledge. However, according to the process undertaken in reviewing this body of literature, only around ten percent of the published studies (excluding those not published/available in English) were considered to warrant inclusion on the basis of their relevance to previously-identified priority research knowledge gaps and their scientific merit: nine in ten studies published during this six year period did not address these knowledge gaps or were not reported in a way that allowed us to be certain of their conclusions. This does not, of course, mean that these studies were without value, but it does highlight the real issue of how animal influenza researchers from across the globe can come together in a way that favours maximum progress in the areas in which it is most needed.

The authors of this report believe that meaningful advances in understanding and controlling animal influenza viruses will require an increasingly "joined up" approach to research/research funding, policy and implementation across geographic and institutional borders, as well as along the bench-to-pen-side innovation pipeline. This report forms an important resource that can be used towards a comprehensive analysis of priority research knowledge gaps, but the success of this process will depend on the effective use and implementation of the ensuing findings by all key stakeholders.

The progress reported here leaves room for considerable optimism about upcoming developments in the animal influenza field in future years; in particular, we feel that the exponential growth in the ability of computer modelling to assist in understanding various aspects of influenza including epidemiology, immunology, vaccine design and outbreak control is especially exciting and should be fully leveraged. At the same time, lessons can, and should, be learned from the SARS-CoV-2 pandemic and various countries' responses to the arrival of the virus at their doors. Broad insights into understanding and control of potentially zoonotic viruses are emerging from this global disaster. In particular, the success of the SARS-CoV-2 research-to-vaccine pipeline should be noted in the animal influenza context and the key role of streamlined, but still standardised, vaccine-testing protocols in identifying lead candidates for further development.

Bringing together policy-makers, researchers and practitioners across both private and public sectors, with the common aim of increasing our knowledge of animal influenza viruses, their hosts and the interaction between the two, holds great promise for future advances in control and understanding of this most enigmatic of viruses.

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Appendices

Abbreviations

AGID	Agarose-Gel Immunodiffusion
AGP	Antibiotic Growth Promoter
AHAW	Animal Health and Welfare panel (of the European Union)
AI	Avian Influenza
AICWG	Animal Influenza Countermeasures Working Group
AIV	Avian Influenza Virus
ANSES	The French Agency for Food, Environmental and Occupational Health & Safety
APC	Antigen-Presenting Cell
APHIS	Animal and Plant Health Inspection Service, USDA, USA
ARS	Agricultural Research Service, USDA, USA
BLP	Bacteria-Like Particles
BPL	Beta-Propriolactone
BSL	Bio-Safety Level
CAAS	Chinese Academy of Agricultural Sciences
CD	Cluster of Differentiation
CDC	Centers for Disease Control and Prevention, US Department for Health and Human Services
cDNA	Complementary Deoxyribonucleic Acid
CEIRS	Centers of Excellence for Influenza Research and Surveillance
CIV	Canine Influenza Virus
COX-2	Cyclo-Oxygenase 2
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
C_T	Threshold Cycle
CTL	Cytotoxic T Lymphocyte
CTLA4	Cytotoxic T Lymphocyte associated Antigen 4
DC	Dendritic Cell
DEV	Duck Enteritis Virus
DIVA	Differentiation of/Differentiating Infected from Vaccinated Animals
DHS	Department of Homeland Security, USA
DNA	Deoxyribonucleic Acid
EID_{50}	50% Egg-Infectivity Dose

EIV	Equine Influenza Virus
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
FDA	Food and Drug Administration, USA
FLI	Friedrich-Loeffler-Institut, Germany
GFP	Green Fluorescent Protein
GISRS	Global Influenza Surveillance and Response System
GMP	Good Manufacturing Practice
HA	Haemagglutinin
HAU	Haemagglutination Unit
HHS	Health and Human Services, USA
H1N1pdm09	2009 pandemic H1N1 Influenza A virus
HI	Haemagglutination Inhibition
HPAI	Highly Pathogenic Avian Influenza
HSPD-9	Homeland Security Presidential Directive Nine
IAV	Influenza A Virus
IBDV	Infectious Bursal Disease Virus
ICS	Immunochromatographic Strip
IDV	Influenza D Virus
IFITM	Interferon-Induced Transmembrane Protein
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB Kinase
IL	Interleukin
iNKT	invariant Natural Killer T (cells)
IVPI	Intravenous Pathogenicity Index
LAIV	Live Attenuated Influenza Vaccine
LED	Light-Emitting Diode
LPAI	Low Pathogenicity Avian Influenza
LPBE	Liquid Phase Blocking ELISA
LPM	Live-Poultry Market
iiRT-PCR	insulated isothermal RT-PCR
IRES	Internal Ribosome Entry Site
IRF	Interferon Response Factor

M	Matrix
mAb	monoclonal Antibody
MBCS	Multibasic Cleavage Site
MDA	Maternally-Derived Antibodies
MDCK	Madin-Darby Canine Kidney
NA	Neuraminidase
NADC	National Animal Disease Center, USDA-ARS, USA
NAHLN	National Animal Health Laboratory Network, USDA, USA
NAI	Neuraminidase Inhibitor
NAPAPI	North American Plan for Animal and Pandemic Influenza
NDV	Newcastle Disease Virus
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGS	Next-Generation Sequencing
NI	Neuraminidase Inhibition
NP	Nucleocapsid
NVS	National Veterinary Stockpile
NVSL	National Veterinary Services Laboratories, USDA-APHIS
ODN	Oligodeoxynucleotides
OIE	Office International des Epizooties (World Organisation for Animal Health)
OFFLU	OIE FAO network on animal influenza
PCR	Polymerase Chain Reaction
PFU	Plaque-Forming Unit
PGE2	Prostaglandin E2
p.i.	post-infection
PLA	Proximity Ligation Assay
PPE	Personal Protective Equipment
RAD	Rapid Antigen Detection
RIG-I	Retinoic acid-Induced Gene I
RNA	Ribonucleic Acid
RPA	Recombinase Polymerase Amplification
RT-LAMP	Reverse-Transcription Loop-Mediated Isothermal Amplification
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
rRT-PCR	Real-time Reverse-Transcription Polymerase Chain Reaction
SA	Sialic Acid

scFv	single-chain variable Fragment
SEPRL	Southeast Poultry Research Laboratory, USDA-ARS
SIV	Swine Influenza Virus
SLA	Swine Leucocyte Antigen
SNP	Single-Nucleotide Polymorphism
SP	Structural Protein
SPF	Specific-Pathogen-Free
SRH	Single Radial Haemolysis
$TCID_{50}$	50% Tissue-Culture Infectious Dose
TLR	Toll-Like Receptor
TRIM32	TRIpartite Motif 32
USD	United States Dollars
USDA	United States Department of Agriculture
VI	Virus Isolation
VLP	Virus-Like Particle
VNT	Virus-Neutralization Test
WHO	World Health Organization of the United Nations
WIV	Whole Inactivated Virus (vaccines)

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Conflict of Interest Statement

The authors declare no conflict of interest.

Additional Resources

Please see the following websites for additional information and/or resources around the content and aims of this report:

https://www.discontools.eu

https://www.offlu.org/

https://immunologicaltoolbox.co.uk