Deliverable 5.5

Methods for prediction of pathogen phenotype from genotype data and structure models

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Contents

Deliverable Description

Deliverable 5.5 follows from Task 5 of Work Package 5 to improve the prediction of phenotype changes related to antigenicity, virulence, transmission, and other traits of pathogens that are relevant for public and animal health. This Deliverable describes methods for prediction of pathogen phenotypes from genotype data and structural models that may be applied – among others – for the research on novel approaches to (re-)emerging disease detection and outbreak response as described in Work Package 8, including the mining of metagenomic data from stool and environmental samples (Task 1), the early detection of emerging zoonoses from wildlife reservoirs (Task 2) and the detection of changes in pathogen traits enhancing risk of outbreaks and pandemics (Task 3).

Output of next generation sequencing (NGS) data in terms of risk analysis is maximized if phenotypic changes of the pathogens can be inferred from the sequences. The CDC "H5N1 genetic changes inventory" (http://www.cdc.gov/flu/pdf/avianflu/h5n1-inventory.pdf) is an excellent example of how – based on all available experimental data – potential phenotypes can be inferred from influenza virus genome sequences and mutations therein. However, it is generally believed that there are multiple evolutionary pathways that would change important phenotypic traits of pathogens. Laboratory studies have for instance implicated convergent evolution of certain molecular determinants for pathogenicity and host specificity for influenza viruses in nature. Under Task 5, we investigated the extent to which functionally equivalent substitutions are found in nature, using model pathogens and phenotypes. Although functional experimental data remain the gold standard when relating sequence changes to phenotypic changes, providing experimental data for each SNP detected in pathogen genome sequences will be unfeasible. We therefore further proposed a computational structural approach to understand the underlying principles for functionally-equivalent substitutions already identified, and to use these to identify possible other functionally equivalent substitutions. Although the initial focus was on existing experimentally derived structures of the influenza HA molecule and influenza virus phenotypes in general, the methods described here will be applicable to other pathogens and phenotypes.

Influenza phenotypes relevant for public or animal health

The WHO Global Influenza Surveillance and Response System (GISRS) was launched approximately 70 years ago to provide early warning signals of changes in influenza viruses circulating in humans around the globe, to reduce the consequences of pandemic influenza, and to maintain desirable efficacy of seasonal influenza vaccines (Hay & McCauley, 2018). Various networks under the auspices of other national and international organizations, including those specialized in animal health (e.g., EU, FAO) also monitor influenza viruses that are circulating in wild and domestic animal populations using passive or active surveillance programs. Some of these programs are long-term and systematic (e.g., poultry, pigs, horses), while others may be more ad-hoc (e.g., HPAI outbreaks in wild birds) or research-oriented (e.g., in bats). In these human and animal virus surveillance programs, viruses are detected routinely by molecular tests and virus isolation. Using the collected viruses, a number of virus properties are characterized that are of relevance to public or animal health.

The virus property that is most frequently characterized is the virus **type** and **subtype**. This phenotype is classically identified using serological tests to discriminate influenza virus types (A, B, C, D) based on lack of antigenic cross-reactivity of the internal proteins (e.g., nucleoprotein or matrix protein), where antibodies against type A viruses do not cross-react with the NP or M proteins of viruses of types B, C and D and *vice versa*. Among the type A viruses, antigenic subtypes can be defined similarly, but now based on antibody cross-reactivity against the hemagglutinin (HA) and neuraminidase (NA) proteins. Antibodies against H1 HA or N1 NA typically do not cross-react with other hemagglutinins (H2-H18) and neuraminidases (N2-N11), respectively, in various serological tests (hemagglutination inhibition, virus neutralization, immunodiffusion), and *vice-versa*. Proper characterization of the virus type and subtype is important for systematic reporting, but even more so to signal unusual infections (e.g., influenza B virus in seals or influenza A/H5 virus in humans), and to discriminate notifiable avian influenza (A/H5, A/H7) from non-notifiable avian influenza in poultry.

Antigenic diversity within a virus subtype (i.e., **antigenic drift**) is particularly important for influenza viruses that are targeted by vaccines (e.g., human influenza, swine influenza, equine influenza, avian influenza in poultry). Influenza vaccines are generally most effective when the vaccine strains are matched antigenically with the circulating strains, which is why, e.g., WHO GISRS monitors antigenic drift of influenza viruses in humans using a global surveillance network. This network and networks with the same aims for animal surveillance may monitor the antigenic properties of evolving strains, the antibody responses in local or global target populations, or vaccine effectiveness directly, in relation to circulating viruses. Virus-specific antibody responses in humans and animals are measured routinely using hemagglutination inhibition assays, neuraminidase inhibition assays, or virus neutralization assays.

Viral **pathogenicity** is another important phenotype. Notifiable avian influenza viruses of the H5 and H7 subtypes can evolve in poultry from **Low Pathogenic Avian Influenza (LPAI)** variants to become **Highly Pathogenic Avian Influenza (HPAI)** variants. HPAI spreads rapidly, causing serious disease with high mortality (up to 100% within 48 hours) in most poultry species. In contrast, LPAI viruses cause generally no or a mild disease, and may easily go undetected. When HPAI variants emerge, measures must be taken as outlined by the World Organisation for Animal Health (OIE). In most countries including those of the EU, measures are taken to stop the spread of the disease, to eradicate it as soon as possible, to ensure the safe movement, imports and exports of animals and their products and to inform trading partners and other interested parties about the actions taken. Moreover, HPAI viruses are on the Select Agent list in the USA and are specified in legislation (e.g., import/export controls) in many countries. The classical test to identify HPAI viruses is by intravenous injection of virus in chickens to determine the intravenous pathogenicity index (IVPI). An IVPI >1.2 in 6-week old chickens is indicative of HPAI virus.

Increased pathogenicity (or virulence) beyond the LPAI/HPAI definition is also important for poultry and other hosts, including humans, but is more difficult to establish and often requires case-reports or epidemiological investigations (e.g., for humans) or experimental infection of laboratory animals. Likewise, alterations in influenza virus **host-range** are of particular interest to signal increased risks of cross-species transmission (zoonotic risks, or transmission to new animal hosts, such as pigs), but also these signals generally follow case-reports and epidemiological observations. In the laboratory, changes in host range have generally been investigated using experimental infections of laboratory animals but surrogate tests with cell lines, tissues, or other host-specific reagents such as embryonated eggs have also been developed. Pathogenicity and host-range may also be linked, e.g., when increased pathogenicity or host-range is caused by alterations in viral **tissue-tropism**. Virus **transmission** (between animals or between humans) is yet another important phenotype that can be linked with pathogenicity, host-range, and tropism but is hard to determine without the use of experimental infections of laboratory animals. Clearly, virus transmission is an important phenotype to characterize, as without efficient onward transmission from an individual infected case the impact of a virus would be limited to one or few infected hosts and outbreaks would die out without intervention.

A final phenotype that is routinely monitored in surveillance studies in animals and humans is **antiviral drug resistance**. Mostly for use in humans, two classes of antiviral compounds are currently on the market – the M2 ion channel inhibitors and the NA inhibitors – but drug resistance can emerge rapidly either upon treatment or spontaneously. Knowing which virus strains or lineages are already resistant is crucial for clinical use of the antivirals, and can be monitored by in-vitro or in-vivo testing of viruses in combination with the antivirals.

High throughput assays to identify new or functionally equivalent phenotypes and genotypes

Although the true phenotypes mentioned above (virulence, host-range, transmission, vaccine-escape, drug-resistance, type-subtype-antigenic variation) are clearly the target of most biological characterizations, some of the required "gold-standard" assays are laborious and often involve experiments with (laboratory) animals. As a consequence, high-throughput surrogate assays have been developed that are indicative – though not always conclusive – for these major phenotypes and are briefly mentioned here.

For instance, given that true vaccine escape is hard to monitor, the influenza community has identified "correlates of protection" that may be determined by high-throughput **virus neutralization assays**, **hemagglutination inhibition assays**, and **neuraminidase inhibition assays**. Most antigenic drift variants have been identified via the use of these surrogate assays and sera from experimentally infected animals, and the molecular basis of antigenic drift – including numerous specific mutations in HA and NA – was largely characterized with these tests (e.g., Koel et al., 2013, 2014). Recently, **enzyme-linked lectin assays** were described for high-throughput measurement of antigenic properties of influenza virus NA proteins (Couzens et al., 2014; Westgeest et al., 2015). **NA enzymatic assays** like the MUNANA or NA-STAR assay are in use to measure neuraminidase drug resistance routinely and resistant genotypes are now frequently established using such tests (e.g., Lackenby et al., 2018).

Given that virulence, tissue-tropism, host-range and transmission are often linked, several assays have been developed that may investigate a few such phenotypes at the same time. For instance, **polymerase activity** may be measured in the form of **minigenome assays** or **plaque assays** to establish host-specificity, tissue-specificity, temperature specificity and overall polymerase activity (Mänz et al., 2016). HA receptor specificity and affinity that are also related to virulence, tissue-tropism, host-range and transmission can be measured in various **HA binding assays** (e.g., solid phase binding, glycan arrays, hemagglutination with erythrocytes of various sources, virus-histochemistry). **HA fusion assays** (e.g., haemolysis and syncytium assays) and **HA temperature stability assays** (agglutination) and have been developed as indicators of potential increased HA stability in terms of pH and temperature, which was identified as a potential determinant of transmissibility between mammals (Linster et al., 2014).

The above assays have frequently served the purpose to identify mutations that can confer a particular phenotype previously linked to a particular strain. Exemplary studies are the identification of mutations that change the receptor specificity of influenza virus HA (e.g., Chutinimitkul et al., 2010, Schrauwen et al., 2016, De Vries et al., 2017), HA stability (e.g., Russier et al., 2016), polymerase activity in mammalian cells (e.g., Mänz et al., 2016), antigenic drift of HA (e.g., Koel et al., 2013, 2014), and influenza virus transmission traits (e.g., Linster et al., 2014). Importantly, these newly catalogued mutations associated with relevant influenza virus traits add to the CDC "H5N1 genetic changes inventory", and are already used routinely to infer traits from newly emerging viruses, such as the European HPAI A/H5Nx viruses (e.g., Harder et al., 2015). When relevant, viral traits may be confirmed in experimental challenge studies in animals such as ferrets (e.g., Grund et al., 2018; Herfst et al., 2018) (see also below).

Prediction of function from structures

Influenza A/H7N9 viruses in China have raised concern for a new influenza pandemic emerging from avian reservoirs. Some of these viruses possess known mammalian adaption markers in the hemagglutinin (HA) and in the polymerase genes but, to date, no sustained human-to-human transmission has occurred. Within COMPARE, we aim to use a computational structural approach to understand the underlying principles for functionally-equivalent substitutions already identified, and to use these to identify possible other functionally-equivalent substitutions.

Structural Stability

The effects of changes in the amino acid sequences of the hemagglutinin gene of A/H7N9 viruses circulating since 2013 have been assessed using a variety of computational techniques. The H103Y (H5 numbering) substitution was previously shown to increase the stability of A/H5N1 viruses. Using computational modeling, a N94K substitution located at the trimer interface of the A/H7N9 A/Anhui/1/13 virus (AN1) HA was identified, which was predicted to have a similar impact as the H103Y substitution, interacting with charged amino acids within neighboring monomers. An A210E substitution, also located at the trimer interface although more distal to the stalk, was predicted to form interactions with the side chains of T156 and S237 on the neighboring monomer, thus increasing its stability. A K58I substitution (HA2 numbering), which is known to increase the HA stability of A/H5N1 and A/H7N1 viruses, was the third substitution investigated. In wild-type AN1, K58 is predicted to interact with nearby N282, causing a kink in the peptide backbone providing less than optimal interactions between the adjacent monomers. The K58I mutation would lead to a loss of this interaction but, surprisingly, is predicted to result in movement of the backbone, improving interactions (and thus stability) of the neighboring charged amino acids R54 and E57 in HA2 to the adjacent monomer.

These three substitutions were assessed for their ability to alter the stability of AN1 experimentally (Schrauwen et al, 2016). The substitution N94K causes an increase in acid stability but the temperature stability was not increased compared with AN1. The K58I substitution resulted in a marked increase in both acid and temperature stability of AN1 HA. AN1-A210E presented a similar pH threshold for fusion as the AN1 and a higher HA thermo-stability. Thus, we identified amino acid substitutions that would alter the HA stability and binding affinity of A/H7N9 viruses, properties that have been shown to be critical for airborne transmission of avian influenza viruses between mammals. These data and predicted molecular mechanisms are useful for surveillance and assessment of the pandemic potential of zoonotic viruses, upon confirmation of the virus phenotypes in appropriate animal models.

Receptor Specificity

Several mutations in the receptor-binding site (RBS) are well established to alter the receptor-binding specificity of avian influenza virus strains to that of human viruses. These include GD225, QL226 and GS228 (H3 numbering), The QL222 substitution was seen in the initial wave of human infections with A/H7N9 viruses in 2013. We have used sequence and structural analysis to predict how mutations within the RBS would alter the interactions of the virus with α2,6 glycans. We have also evaluated sequence changes within this region of the HA that have been seen in recent A/H7N9 viruses which may further increase binding to human receptors. The only mutation within the RBS seen in recent A/H7N9 viruses is GD225, which is present in five viruses. Continued monitoring of sequence changes potentially altering receptor specificity is ongoing.

Antigenic change

Antigenic cartography was used on HI data extracted from publicly available WHO reports. We observed some antigenic variation in viruses circulating after the 4th wave (2015/2016) of A/H7N9 viruses. It has previously been demonstrated that amino acid substitutions at seven positions (145, 155, 156, 158, 159, 189 and 193; H3 numbering) cause the major antigenic changes in many subtypes of influenza virus (Koel et al. 2013, Koel 2014). These are located on the exposed surface of the HA, peripheral to the RBS. These substitutions in general cause large changes in the biophysical properties (volume & charge) of their side chains, disrupting antibody interactions with the virus. We have used sequence and structural analyses of the recent A/H7N9 sequences to identify substitutions around the rim of the RBS that produce large changes of amino acid properties that could be responsible for these antigenic differences. Mutations at three of the seven positions have been seen in recent A/H7N9 viruses. Further work is ongoing to validate the genetics of the observed antigenic differences.

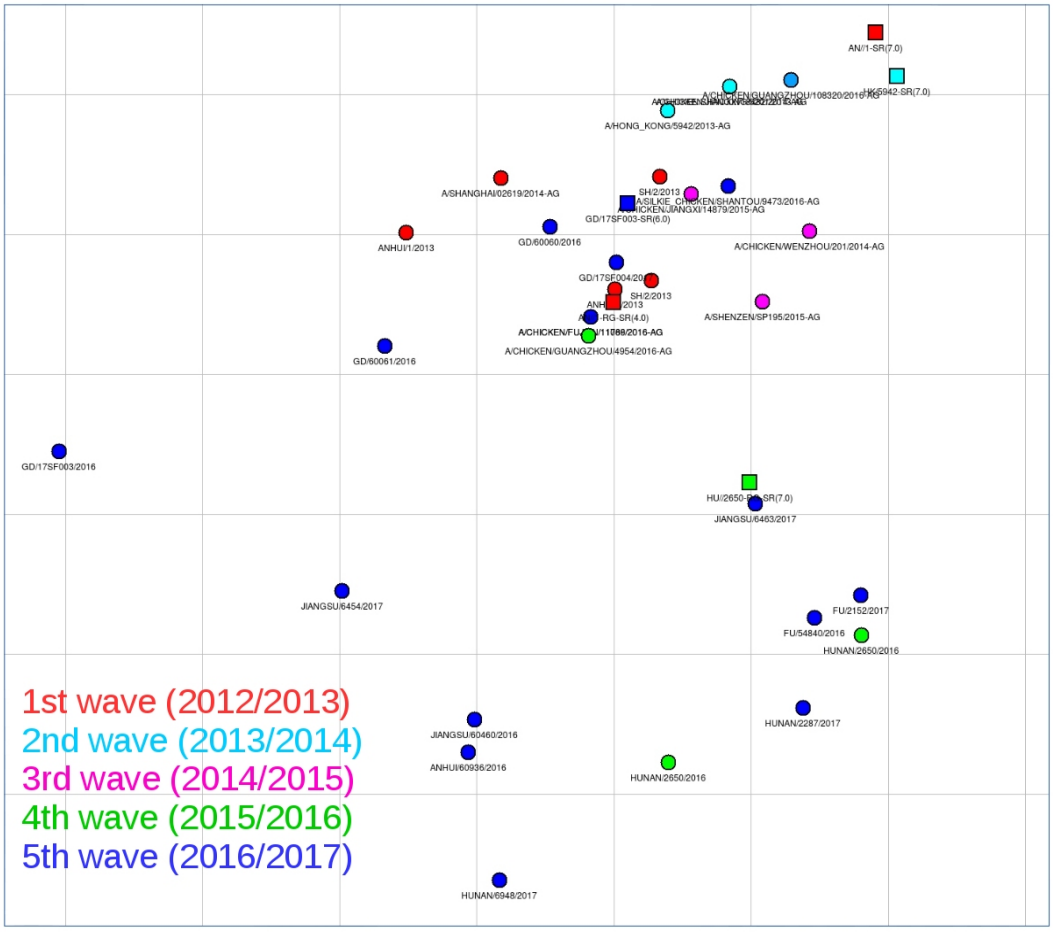

Figure 1. Antigenic map of emerging A/H7N9 viruses, based on hemagglutination inhibition assays with ferret antisera.

Validation in experimental models

The validation of the sequence-based isolate or strain variations in different animal models is a central part of characterizing variant influenza viruses. It is also the main experiment for validating sequence-based and structure-based predictions concerning replication or virulence properties of the different influenza virus variants or reassortants. For Influenza A viruses (IAV), a broad spectrum of animal models is available and can be used. Within COMPARE, the major animal models such as chicken, ducks, pigs, mice and ferrets are available and are utilized for virus characterization.

As a first example, the emerging H5N8 clade B viruses were genetically analyzed (e.g., Pohlmann et al. 2017) and numerous virus variants and reassortants detected. The selected HPAIV H5N8 clade B variant was comparatively tested in different animal models (chicken, Muscovy ducks, Pekin ducks, Balb/C-mice and ferrets). It was demonstrated that (1) the H5N8 viruses are highly pathogenic with an intravenous pathogenicity index in chickens of 2.8-3.0, (2) a high virulence for both Muscovy and Pekin ducks, (3) and high virulence also shown in the Balb/c-mouse-model, but not in ferrets (Grund et al. 2018). The experiments therefore confirmed the high virulence of the H5N8 clade B virus in ducks, but it was also shown that the zoonotic potential of the tested strain is low. This phenotype in ferrets was also confirmed for related HPAIV H5-strains from Germany (i.e., H5N5 and H5N6 viruses). Both viruses were highly pathogenic in chickens, but like the H5N8 clade B virus, there was a very mild course of infection in the ferret model.

In a second example, APHA have investigated the interspecies transmission and serial passage of a clinical HPAIV H5N8 virus isolate from ducks. Ducks were experimentally infected and transmission to ducks, turkeys and chickens, followed by serial transmission to two further groups of birds of the same species, was assessed (Puranik et al., ‘Transmission properties of H5N8 HPAIV (2014, clade 2.3.4.4) from infected donor ducks to contact ducks, chickens and turkeys with the identification of emerging genetic changes‘, in draft). Mutations leading to a change in the amino acid structure of the virus were present in samples from the second and fourth stages of transmission, which may be indicative of viral adaptation to different avian species. Analysis of the quasi-species/virus population composition of virus in experimental samples from the respective host species is ongoing in conjunction with Compare WP8.

Application to other pathogens

For pestiviruses, NGS sequencing of virus isolates coming from severely diseased cattle in North Rhine Westphalia and Lower Saxony (Germany) revealed the coexistence of three distinct genome variants within recent highly virulent bovine viral diarrhea virus type 2 (BVDV-2) isolates. While the major portion (ca. 95%) of the population harbored a duplication of a 222-nucleotide segment within the p7-NS2-encoding region, the minority reflected the standard structure of a BVDV-2 genome. In addition, unusual mutations were found in both variants, within the highly conserved p7 protein and close to the p7-NS2 cleavage site. Using a reverse genetic system with a BVDV-2a strain harboring a similar duplication, it could be demonstrated that during replication, genomes without duplication are generated de novo from genomes with duplication. The major variant with duplication is compulsorily escorted by the minor variant without duplication (Jenckel et al. 2014). Finally, these results suggest that the variant with duplication plays the major role in the highly virulent phenotype.

In case of African swine fever (ASF), outbreak characteristics of wild boar varied considerably between the southern and the northeastern part of Estonia. In contrast to high mortality and mainly virus-positive animals observed in the southern region, mortality was low in the northeastern area. In the latter, clinically healthy, antibody-positive animals were found in the hunting bag and detection of virus was rare (Nurmoja et al. 2017). A follow-up study using animal trials providing NGS-sequenced full-length genome sequences revealed an attenuated phenotype to be responsible for the mild ASF infection of wild boars in the northeastern part of Estonia. In the genome of the attenuated ASF variant, 14,560 bp at the 5’ end are missing, resulting in the loss of 26 complete genes, and genome reorganization by duplication was detected (Zani et al. 2018). In conclusion, an ASFV variant was found in Estonia that showed reduced virulence.

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